KIDNEY TRANSPLANT; GRAFT AND RECIPIENT PROFILING

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ABSTRACT

Despite the recent introduction of a number of new and more potent anti-rejection drugs, the incidence of rejection and long-term graft survival remain unchanged. There remains a significant difference in long-term graft survival depending on the source of the donor. The purpose of this study was to examine gene expression in the transplanted kidney using microarray technology to identify potential biomarkers that could be used to predict and monitor graft function so that appropriate interventions could be made in the event of graft dysfunction.

Over a 5 year period RNA was extracted from 144 donor kidneys that were transplanted. The initial attempts at probe preparation and hybridization were unsuccessful. This led to the development of a new strategy which involved the use of state-of-the-art microarray technology which embraced the advances realised with the completion of the human genome project. Microarray data was analysed using J-Express and Pathway studio. Significance analysis of microarray, hierachical clustering, gene ontology mapping and pathway analysis was performed.

The identification of potential biomarkers that had previously been described by other authors validated this approach. In addition novel genes were identified that may have a role as biomarkers of graft function. Other potential biomarkers were identified that represented cellular processes that could be modified by therapeutic intervention thus possibly changing the clinical outcome or allowing monitoring of the success of therapy.

Confirmation of previously described biomarkers and the identification of novel potential biomarkers has confirmed that gene expression profiling has a valuable role in identifying processes that are indicative of disease processes including those involved in kidney transplantation. Furthermore with the development of minimally invasive tests to measure these biomarkers, we can potentially change the natural history of the disease process, and hence, preserve graft function and possibly prolong life.

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LIST OF ABBREVIATIONS

aa	Amino acid
AMV	Avian myeloblastosis virus
aRNA	Antisense RNA
BASE	BioArray Software Environment -
dsDNA	Double stranded DNA
CTLA	Cytotoxic T cell lymphocyte-associated antigen
ELISA	Enzyme-Linked Immunosorbant Assay
Fab	Fragment antigen-binding fragment
GAL file	GenePix Array List Files
GVHD	Graft versus host disease
HLA	Human Leukocyte Antigen
ICE	(IL-1β converting enzyme)
IFNγ	Interferon γ
IL	Interleukin
MAC	Membrane attack complex
MAIME	Minimum Information About a Microarray Experiment
MASP	Mannose associated serine protease
MBP	Mannose-binding protein
MBP	Major basic protein
MHC	Major Histocompatability complex
mRNA	Messenger RNA
NHS	N-hydroxysuccinimidyl
NK	Natural killer
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophil
RNA	Ribonucleic acid
TNF-α	Tumour necrosis factor α
TGF-β	Transforming growth factor β
TIFF	Tagged Image File Format

1 INTRODUCTION

Graft Survival in Kidney Transplantation

Despite the recent introduction of a number of new and more potent anti-rejection drugs the incidence of rejection and long-term graft survival remain unchanged.

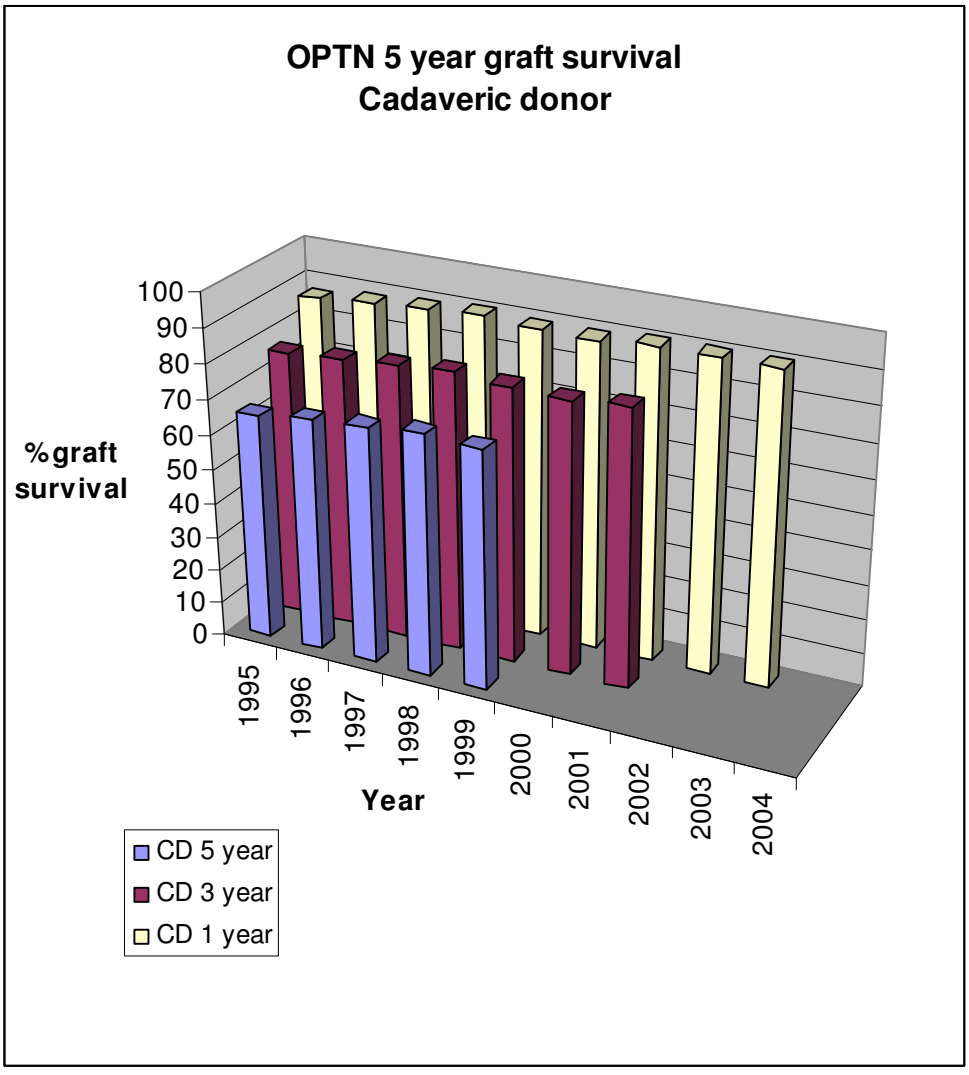

Figure 1 - OPTN five year graft survival (deceased donor)

Figure 1 demonstrates Kaplan-Meier Graft Survival Rates for Transplants performed between 1995 and 2002 [1]. Figure 2 shows the equivalent data for graft survival of living donor grafts.

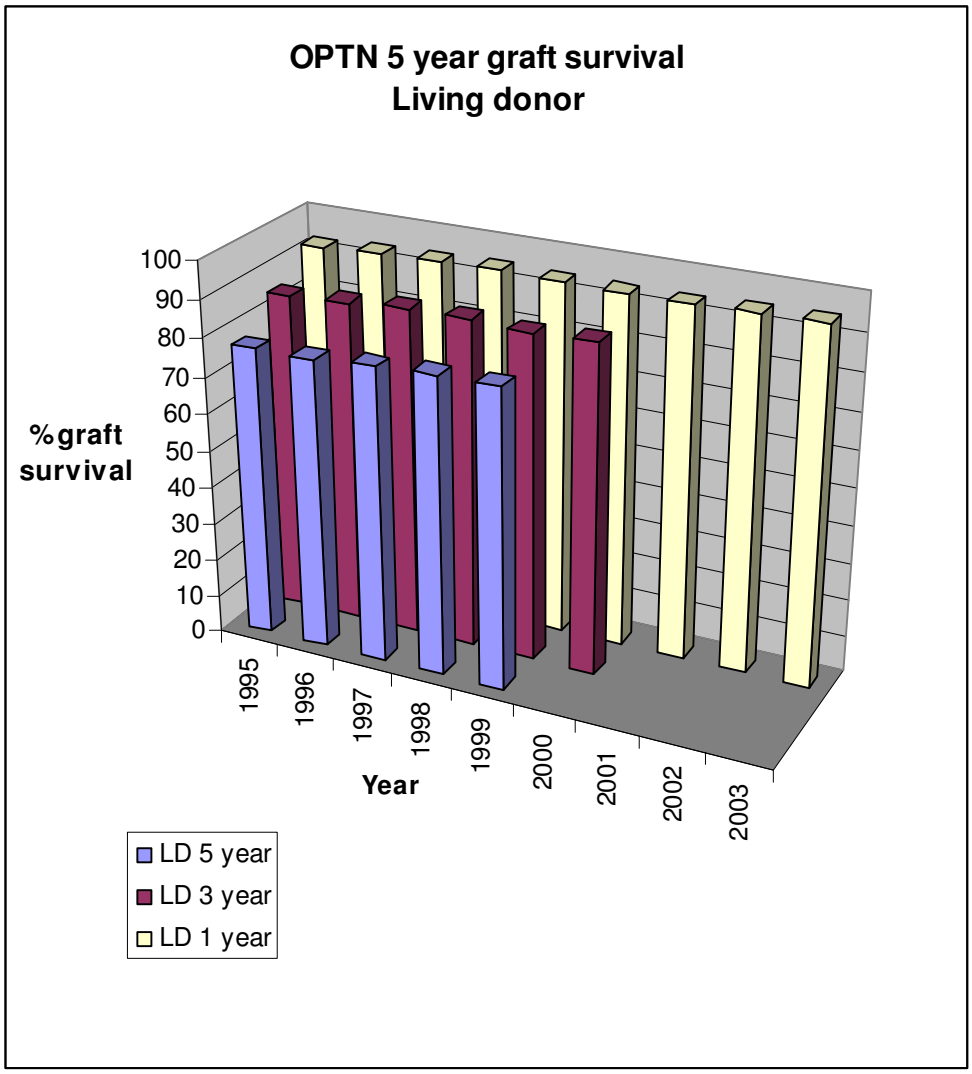

Figure 2 - OPTN five year graft survival (living donor)

For both deceased and living donors these plots show that graft survival at 1, 3 or 5 years has remained constant between 1995 and 2002. Approximately 90 % of deceased and 95 % of living donor grafts survive at one year. Approximately 69 % of deceased and 79% of living donor grafts survive at 5 years [1].

At the same time the number of kidney transplants performed each year remains about the same but the demand for transplantation i.e. the number of patients on the transplant waiting list continues to steadily rise (see figure 3). The reduction in numbers of organs made available from deceased donors is

being balanced by an increasing number of living donors. Non-heart beating donors expand the donor pool and current legislation now supports altruistic and paired organ donation [2]. These more recent forms of donation help the "demand and supply" mismatch but fail to make a significant impact on the transplant waiting list.

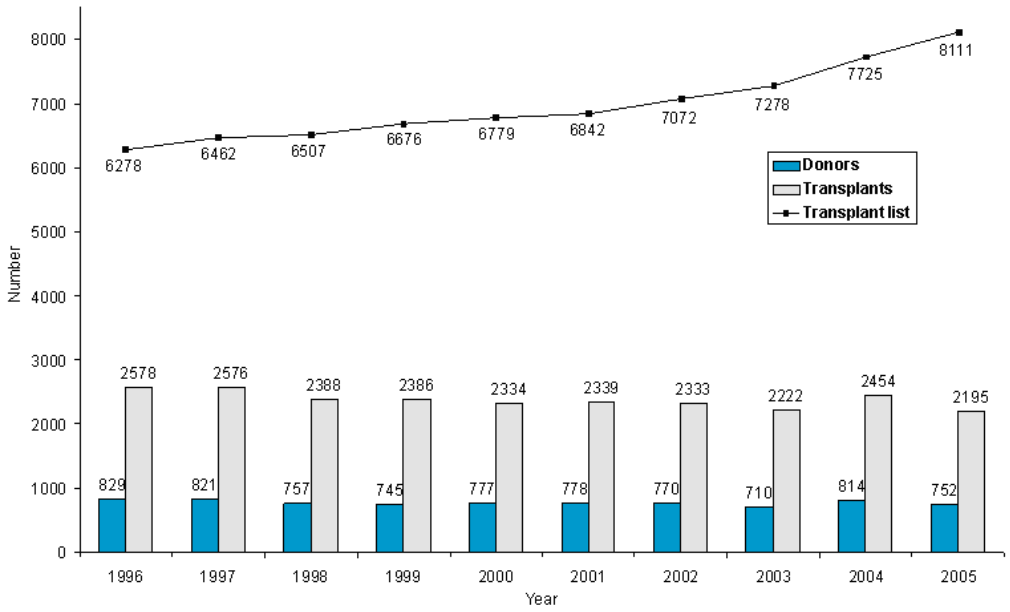

Figure 3 - Number of deceased donors and Transplants in the UK, 1996 – 2005 and Patients on the active and suspended transplant lists at 31 December 2005

It is therefore imperative that when organs become available for transplantation, they are optimally matched to the recipient in an environment that is likely to determine a favourable outcome.

1.1 Factors that influence the outcome of transplantation.

1.1.1 Donor Factors

Donor factors include a host of variables some of which are relatively constant (e.g. gender, blood group, donor category) with others being highly dynamic (Cold ischaemic time, age, blood pressure); there are also the unknown factors. This study will aim to produce results that corroborate previously identified biomarkers and hopefully discover those that are unknown or have not

previously been implicated in the pathological processes that lead to graft dysfunction.

I. Constant Factors

a) Age

Transplantation outcomes were analyzed in 598 patients who received renal transplants from 1979 to 2002. In the elderly donor group (age >50), graft survival was 92.8% at 1 year; in the younger donor group (age<50), they were 93.4% (P= 0.02). Recipient age >50 years was not found to be an independent risk factor [3]. Pessione found results to suggest that cerebrovascular cause of death, history of hypertension, and elevated creatinine are significant independent donor risk factors for graft survival, whereas donor age was a statistically significant but dependent risk factor [4]. This result is important for the design of allocation and transplantation strategies for kidneys procured in elderly donors. Morrissey *et al* found that graft survival at 1, 2, and 3 years, censored for death with graft function, did not differ when comparing older vs. younger donors [5]. In living donation donor age was found to have no impact on graft survival, however, function at 3 and 12 months was reduced in the age 65+ kidneys [6].

b) Gender

Oh *et al* found that the 'recipient' gender may be more important than the 'donor' gender for early graft function after adult living donor kidney transplantation. The gender-related differences in post-transplant serum creatinine and urine creatinine of recipients were associated with the differences in the parameters of metabolic demands of recipients rather than with the weight of implanted kidney or with the pre-operative renal function of the donor. The early graft function was not determined by donor gender. The effect of recipient gender on the graft function depended on the metabolic demands, which are higher in male recipients. The effect of recipient gender on early graft function depended on the metabolic demands which, on average, were higher in male recipients [7].

c) Blood group

Generally blood group has been accepted as a barrier to transplantation that should not be crossed. This barrier has been successfully crossed in living donation where the use of plasmapheresis and splenectomy has been used [8, 9]. More recently Ishida *et al* successfully used an immunosuppressive regimen for ABO incompatible recipients, including the use of anti-CD20 and anti-CD25 antibodies (Rituximab and Basiliximab) as an alternative to splenectomy [10]. With respect to rhesus status Osman *et al* found that the Rh(D) blood group system was not a clinically relevant histocompatibility barrier to living donor renal transplantation [11].

d) HLA Mismatch

The outcome of a particular allograft is also influenced by the degree of MHC match between donor and recipient. This has been shown in the rat model where very different outcomes occur following liver transplantation depending on the combination of strains used. In DA to Lewis rat liver transplantation, rapid severe acute rejection occurs, whereas Lewis to DA results in spontaneous tolerance. These pure-bred strains are termed high and low responders, respectively [12]. Farges *et al* analyzed the cell infiltrate and cytokine expression in these two models, but found very little difference. The cell infiltrate in each case was the same and the only noticeable difference was a reduction in IL-4 production in the tolerant group.

HLA Class II typing in was introduced in 1980 and there were general improvements in tissue typing techniques for transplants after 1987. Terasaki *et al* found that the 0 ABDR-mismatched grafts had a projected 20-year survival of 40%. This fell progressively by 13% for each mismatch (up to 6) [13].

II. Dynamic Factors

a) Hypertension

Pre-existing long-standing hypertension in the donor was a strong independent variable affecting both DGF and graft function in deceased kidneys at 1 year [14]. Compared with controls, duration of HTN was an independent risk factor

for graft survival. 3-year graft survival rates were 75% versus 65%; relative risk = 1.36 in the hypertensive group ($P < 0.001$) [15].

b) Brain-Stem death

Brain-stem death usually occurs as a result of raised intra-cranial pressure as a consequence of an intra-cerebral bleed. It is well recognised that brain death induces an autonomic storm inducing cytokine release. This manifests as bradycardia, hypertension and irregular breathing in spontaneously breathing patients (Cushing reflex), followed by tachycardia, hypertension, vasoconstriction and high plasma concentrations of catecholamines [16-18]. This can cause renal injury and has a deleterious effect on renal function [19]. Pratschke *et al* postulated that antigen independent factors may influence the rate and intensity of host response following transplant [20]. He observed that insults occurring around the time of organ transplantation become risk factors for allograft failure and suggested that graft injury may be programmed even prior to transplantation. Donor risk factors such as age, cause of death, donor management, consequences of ischemia / reperfusion injury and brain death trigger the graft into an immunologically active state [21].

c) Cold ischaemia time (CIT)

In a rat mode Tullius *et al* found that donor age and duration of cold ischemia acted in a synergistic manner. Cold ischaemic times were of particular relevance when grafts from older donors were used [22].

d) Warm ischaemia time (WIT)

Shiroki *et al* found that prolonged warm ischemia caused a chronic ongoing process, leading to late graft failure in kidneys transplant from Non-Heart Beating donors (NHBDs). They postulated that the response to prolonged ischemic injury may increase graft immunogenicity and promote the chronic changes seen in chronic rejection. Kidney grafts with an extremely prolonged WIT had significantly poorer long-term survival [23].

1.1.2 Recipient Factors

a) Age

Older patients have a lower risk of acute rejection but a poorer death-censored graft survival. This result is not explained by any combination of patient age with donor age, delayed graft function or immunosuppression [24].

b) Underlying cause of renal failure

The aetiology of renal failure may have an impact on the graft outcome. For example, focal segmental glomerulosclerosis (FSGS) is a condition in which the filter units within the kidney are damaged and lead to proteinuria. In the more severe cases end-stage renal failure ensues leading to the need for dialysis of a kidney transplant. The overall recurrence rate of FSGS in the transplanted kidney is 30% and is associated with increased rate of graft loss [25-27].

c) Pre-existing medical conditions, co-morbidities and performance status

Clearly a younger transplant recipient who has had a short lived primary disorder is more likely to have a better prognosis than a more elder recipient with for example the long term consequences of diabetes mellitus, ischemic heart disease. Performance status is an objective system of measuring a patients overall fitness for any medical procedure[28]. This measure has not been used in relation to kidney transplantation and prognosis.

d) Immunosuppression

Variable pre-dose cyclosporin concentrations may be a risk factor for graft loss [29]. The calcineurin inhibitors are known to be nephrotoxic at high concentrations. Long term steroid use has well documented long term sequelae. It can be seen that poly-pharmacy associated with immunosuppression and the treatment of concurrent conditions can all have detrimental effects on graft function and the overall health of a transplant recipient. The analyses of the effects are combinations of immunosuppressants and other medications and their effect on long term graft and patient survival are outside the scope of this study.

e) Hypertension

Early hypertension is common after renal transplantation. Early BP control has the potential to influence the risk of allograft rejection and delayed graft function[30]. Early post-transplantation systolic hypertension strongly and independently predicts poor long-term graft survival in paediatric patients[31].

There are clearly many donor, recipient and environment variables that interplay to result in the immune response. Some of these factors have a common influence upon the immune response that manifests itself in the form of acute rejection; a variable phenomenon which is usually has a detrimental effect on the function of the graft.

1.2 Acute rejection

The effector limb of rejection consists of the mechanisms concerned with actual graft damage and ultimate destruction and may be divided into the cell-mediated or the antibody-mediated (humoral) response.

1.2.1 Acute cellular rejection

Cell-mediated graft destruction occurs once the inflammatory cell infiltrate has migrated into the graft. The mechanism involves CD8 + T cells which exert their cytotoxic effects following the recognition of foreign, endogenous antigen in association with the MHC Class-I molecule by the T-cell receptor. Toxins, such as Fas ligand and serine esterases are released, as are a group of molecules called ‘perforins’. The perforins polymerise and form pores in the membrane of the target cell, enabling granzyme, a protease which lyses cytoplasmic components, and other toxins to enter. Although the cytotoxic lymphocytes are capable of significant damage, recruitment of other non-specific cells, such as leucocytes and natural killer cells produces the epithelial damage seen in organ transplant rejection (e.g. tubulitis) with similar inflammatory changes detectable in the endothelium of the graft vasculature.

1.2.2 The humoral response

The humoral immune response is based on the maturation of B-lymphocytes and the production of antibodies (immunoglobulins (Ig)), bi-functional molecules which bind antigen, via their Fab region, and activate further immune functions via their Fc region. There are five Ig isotypes (IgG, 1gM, IgA, IgD and IgE), each consisting of heavy chains, encoded on chromosome 14, and either κ or λ light chains, encoded on chromosomes 2 and 22, respectively. Each chain is composed of one variable domain, forming the antigen binding site, and constant domains.

B-cells produce the huge variation in antibody specificity like that of the T-cell, by the rearrangement of Ig genes at the D (diversity), J (joining) and V (variable) regions. Following this rearrangement, the immature B-cells express the 1gM isotype on their surface with resultant mature B-cells expressing both 1gM and IgD antibody receptors of the same specificity. These mature cells circulate the lymphatics if they fail to interact within a few days with the antigen for which they are specific; they are removed by the spleen. If, however, the surface Ig receptors come into contact with antigen, the B- cell undergoes differentiation, with T-cell help, becoming either the antibody-producing plasma-cell (under the influence of IL-6) or a memory B-cell. Memory cells arise under the influence of IL-5 and circulate, expressing high-affinity antigen receptors, enabling the rapid production of antibody on future encounter with the specific antigen. If antigen cross-links the surface Ig receptors, the B-cells increase in size, undergo DNA synthesis and the antigen is internalised and re-expressed as exogenous peptide in association with MHC Class-II; the B-cell is functioning as an APC.

Antibodies which are damaging to the transplanted organ are of the cytotoxic IgM and IgG isotypes, effective activators of the classical complement cascade. Following antigen binding, the Fc region of the antibody binds and initiates the soluble serum proteins of the complement cascade. These subsequently form the C3- and C5- convertase enzymes and the final, lytic membrane attack

complex (MAC which is similar to the perforins released by cytotoxic lymphocytes.

In renal transplantation, antibodies specific for HLA Class I molecules are clinically significant (see Tissue-typing and cross-matching) and reports of antibodies specific for endothelial cell molecules causing graft loss, even hyperacute rejection, are accumulating [32]. The role of antibodies post-transplantation, in acute rejection, remains controversial. The established view is that antibody is unimportant, the dominant pathway of damage being exerted by the cytotoxic lymphocytes. However, in at least one rat combination, antibody has been shown to b capable of causing acute rejection in the absence of cytotoxic lymphocytes. There has also been recent data linking antibody with a possible role in chronic rejection[33]. It has been suggested that current immunosuppression by targeting T helper 1 responses may cause a natural deviation of the immune response to the T helper 2 pathway and antibody production [34, 35].

It is important, therefore, to minimise the mismatch in HLA class I and II molecules in an attempt to diminish potential adverse reactions. This is partially achieved with tissue typing and cross-matching.

1.3 Tissue typing and cross-matching

HLA (Human Lymphocyte Antigen)

The hypothesis that matching the HLA Class-I and Class-II status of both donor and recipient would reduce the immunogenicity of the graft and, therefore, the number and severity of rejection episodes, led to the introduction of pre-transplant tissue typing. In kidney transplantation, the current practice is to type and match for HLA-A and B (Class-I) and HLA-DR (Class-II). Because each individual possesses two specificities for each, this means matching for six alleles. Large population studies, have shown improved long-term survival when five or six of the HLA antigens are matched [35]. The benefit of matching for liver and cardiac transplantation is much less clear-cut

and currently no prospective matching, other than blood group ABO, takes place for these organs.

a) Lympho-Cytotoxicity Test

The basic serological test used in the tissue-typing laboratory is the lympho-cytotoxicity test (LCT) whereby complement fixation with resultant cell lysis indicates the presence of specific antibody or antigen. In order to determine a tissue type, antibodies (monoclonal or polyclonal) of known specificity are incubated with lymphocyte fractions harvested from peripheral blood. T cells, which constitute 85% of peripheral blood lymphocytes (PBLs), are used to type for Class-I antigens and are separated from the non-T-cell /B-cell fraction, expressing Class-II, by either sheep red blood cell rosetting or density gradient techniques.

b) ABO Blood Groups

In kidney transplantation ABO matching is insufficient because rapid rejection responses due to circulating cytotoxic donor-specific HLA-antibodies can cause an equally damaging effect. In the mid-1960s, the cross-match was introduced into kidney transplantation whereby donor lymphocytes, harvested either from peripheral blood lymphocytes or from the spleen, are incubated with serum from the potential recipient. In addition to this immediate pre-transplant test, the antibody profiles of future recipients may be constructed by incubating sequential serum samples against a panel of HLA-typed lymphocytes.

c) Panel-Reactive Antibody

This panel-reactive antibody (PRA) screening determines the presence and specificity of HLA-alloantibodies and is, therefore, useful in organ allocation. The original LCT principle is still commonly applied to both the cross-match and PRA but sensitivity and specificity have been improved by the development of flow-cytometric and immunoassay methods.

These technologies, although primarily developed for the antibody assessment of kidney transplant patients, have been used to investigate whether donor-specific HLA antibodies are deleterious in other transplant settings. Hyperacute rejection is rare in heart and almost unknown in liver transplantation despite 7-33% of waiting-list patients known to harbour cytotoxic HLA-antibodies [36]. The ability of the liver to counteract the presence of cytotoxic allo-antibodies has been shown to extend to protection of a kidney graft even in the presence of a positive crossmatch if combined liver and kidney transplantation is performed. The mechanism of this protection remains unknown. One hypothesis is based on the production by the liver of a soluble form of MHC Class I molecules, which has been postulated to play a role in protecting the liver by swamping the recipient's immune system with foreign antigen. This may be one of the mechanisms that help to explain the phenomenon known as tolerance.

1.4 Tolerance

When applied to an allograft the definition of tolerance is the co-existence of a transplanted organ or tissue within a recipient without the need for continuous, long-term immunosuppression. This is specific with the immune system being otherwise unimpaired in its protective functions, but the graft is not necessarily free of inflammatory cells. It is also durable, being unaffected by other unrelated stimuli which may activate the immune system of the recipient. Achieving tolerance in a clinical setting would be the 'holy grail' of transplant immunology which clinicians and scientists are working There are some recent clinical trials, which have come close to achieving it[37], and there are many reports of tolerant patients from historical series[38].

i. Deletion

Deletion (with or without chimerism) occurs in a manner similar to the development of self-tolerance. The term 'chimerism' is used to describe a state where donor cells survive long-term in the recipient so that the recipient's immune system becomes unresponsive to the donor cells after deletion of the reactive donor clone.

ii. Co-stimulatory blockade

Blockage of co-stimulation has been reliably achieved using a variety of monoclonal antibodies to co -stimulatory receptors or their ligands such as CD28 and CD40 Examples include CTLA4-Ig, which binds to B7.1 and 2, and anti-CD154 (CD40 ligand) which blocks T-cell interaction with CD40-expressing B-lymphocytes. This T-cell mediated suppression has been shown to be transferable to naive animals. The presence of a graft has been shown to render the naive cells tolerant rather than simply suppressed

iii. Development of regulatory cells.

Sir Peter Medawar was awarded the Nobel Prize for his experiments demonstrating long-term donor-specific tolerance by injection of bone marrow into neonatal mice. After falling into disrepute as a theory in the 1980s, recent data has convincingly shown that it is possible, in certain circumstances, to induce the formation of suppressor cells which prevent rejection from occurring.

Tolerance induction in experimental animal models has a long history. There are many examples of tolerance induction in animal models by these three methods. In order to achieve tolerance it is likely that a combination of these methods will need to be used. Sir Peter Medawar achieved tolerance in his initial experiments because he used neonatal animals. The transplanted bone marrow populated the thymus together with the recipient's native bone marrow; leading to the elimination of donor reactive T-cell clones in the same way that self-tolerance is achieved.

Another mechanism for tolerance induction is by overwhelming the immune response by donor antigen. This is based on the observations in liver transplantation where an allograft can be accepted when there is a full MHC mismatch[39], and the ability of the liver to partially protect a kidney from cytotoxic antibodies in a combined transplant[40]. Evidence from multiple heart and kidney transplants in one animal suggests that the T-cell response can

be turned off by overwhelming the system with large amounts of antigen, leading to apoptosis of the reactive T cell clones.

One result of this extensive experience with the animal models is that it has become clear that tolerance induction is dependent on lymphocytic infiltration of the allograft. This has given rise to the idea that many current immunosuppressive regimens using calcineurin blockers may actually hinder the development of tolerance.

Tolerance reflects a modification in the host's immune response to transplanted tissue. Development of a tolerant state requires the otherwise immunogenic graft to be accepted by the host by one or a combination of the mechanisms proposed above. Factors are required, therefore, to interact between the graft and the host in order to modify the immune response. These factors are known as cytokines

1.5 Cytokines and chemokines

The role of the soluble mediators released by a variety of cells during rejection was relatively under-studied for a long time because of the complexity of the interactions involved. Much new knowledge has been gained recently by applying new molecular techniques such as polymerase chain reaction (PCR) and in situ hybridization to characterize the cytokines and chemokines produced during rejection.

1.5.1 Cytokine Classification

Cytokines are small soluble proteins, and exert their effects by binding to specific receptors on the surface of their target cells. These produce functional changes brought about by protein synthesis. They are involved in the activation and regulation of the immune response to a stimulus. In transplantation, important cytokines include interleukins (e.g. IL-1, IL-2, IL-4, IL-6, IL-9, IL 10, IL-15) and the interferons (e.g. IFN-γ). Certain cytokines, which play an important role in chemo-attraction, are termed chemokines (e.g. RANTES).These can be divided into four subfamilies distinguished by

positions of N-terminal cysteines: C, CC, CXC and CXXXC, the two important ones being the CXC chemokines (e.g. IL-8) and the CC group (RANTES, MCP-1, 2). The CXC chemokines induce migration of neutrophils / monocytes / T-lymphocytes and other cell types whereas the CC group predominantly stimulates and attracts monocytes/ macrophages and T-cells [41]. CXXXCR1 is unique among chemokine receptors in functioning directly as a cell-cell adhesin, CX3CR1 mRNA is expressed at highest levels in brain, but it is found in all organs. The only known ligand is fractalkine. There is only one member of the C family, XCR1 (also known as GPR5). The receptor is specific for the T lymphocyte directed molecule lymphotactin. It was postulated to have a potential role in cancer therapy due to the ability of lymphotactin to synergize with IL-2 in producing an anti-tumour immune response [42].

1.5.2 T-cell growth factors (Interleukins)

A group of cytokines of particular interest in transplantation is the T-cell growth factors (TCGFs) IL-2, 4, 7, 9 and 15. IL-2 is produced by activated T-cells and causes proliferation and differentiation of both T- and B-cells. Other immune cells, such as NK and dendritic cells, also express a receptor for IL-2 although the precise consequences of this interaction are not fully understood IL-2 expression is regulated at the transcriptional level and is blocked by calcineurin inhibition which is the basis by which the most effective current immunosuppressants cyclosporin and tacrolimus prevent rejection. Stimulation of T-cells without the ability to produce IL-2, or where the ability bas been blocked by monoclonal antibody can result in a state of anergy or unresponsiveness. IL-4 is produced by TH cells, basophils and mast cells and is important for TH differentiation. IL-9 is produced by TH cells and is a potent growth factor for cultured but not fresh primary T-cells, suggesting that its activity may be related to the activation state of the T-cell. IL-7 has a pivotal role in intrathymic T-cell development. IL-15 possesses very similar biological ties to IL-2, but crucially is produced by cells other than T-lymphocytes (activated macrophages, epithelial and endothelial cells), and is not blocked by calcineurin inhibitors. It is regulated at both transcription and translational levels.

TCGFs play a critical role in regulating multiple aspects of immune activation, which have been investigated using mice with single gene knock-out (KOs). For example, IL-2 knock-out animals are not immunodeficient by developing lymphoproliferation and autoimmunity. Mice lacking IL-7, which is crucial to T-cell maturation, develop both immunodeficiency and lymphopaenia. In contrast to IL-2, IL-15 KOs develop lymphopaenia and impaired lymphocyte activation, it therefore appears that IL-15 is involved in the normal homeostasis of peripheral lymphoid tissue.

There is a large degree of redundancy in the system. IL-2-deficient mice can mount a vigorous acute rejection response, and the same applies to blockage of IL-4, 7, and 15. IL-2 also plays an important role in priming activated T-cells to undergo apoptosis, and therefore it may also have paradoxical role in the acquisition of peripheral tolerance. The use of calcineurin based immunosuppression may potentially reduce the chance of tolerance occurring.
Cytokines also regulate the production and function of each other; IL-4 antagonizes both the proliferation and differentiation of B-cells and lymphokine-activated killer cells by IL-2. IL-10 prevents IFN-γ and IL-2 synthesis but this is an indirect effect through macrophages. This complicated cross-regulation may be helpful in controlling rejection and may have a possible role in the induction of tolerance.

Variation in the response to disease between a delayed-type hypersensitivity and humoral response has been related to different patterns of cytokine production. Originally demonstrated in the mouse different immune responses have been linked to specific T-cell clones. A T-helper 1 response is characterized by the production of IL-2, IFN-γ and TNF-13 and is seen when delayed-type hypersensitivity predominates. A T-helper 2 response is associated with IL-4, IL-5, IL-6 and IL-13 and is seen when antibody production is predominant. The T-helper 1 cytokine profile has been associated with acute rejection. In humans, the picture is less clear and the type of cytokine profile that produced is dependent upon the type of stimuli and many environmental factors surrounding each immune event. Naive T-cells have the

ability to produce either pattern of cytokines, so that differentiation into T-helper 1 and T-helper 2 subsets is not fixed in an individual cell. It appears that the method of antigen presentation and the mix of other stimulatory factors determine whether a T-helper 1 (primarily associated with cell-mediated immunity) or a T-helper response 2 (anti-body production) occurs.

1.5.3 Chemokines

Chemokines (especially RANTES) have been shown to have an important role. It is expressed 3 days after T-cell activation and is a chemo-attractant for monocytes, and memory T-cells. It results in the accelerated formation of the immune over a larger area. It has been shown to be involved in the recruitment of monocytes and T-lymphocytes in acute rejection, being produced by fibroblasts and tubular epithelium after activation by IL-1 and TNF-α produced by macrophages. There is also some evidence that it plays a role in the development of chronic rejection as it is present in large amounts in the early stages of chronic rejection.

Clinically, cytokine levels are difficult to interpret because their importance lies in the local environment so that systemic levels may not accurately reflect what is occurring locally. They are measured by ELISA (enzyme-linked immunosorbant assay) which gives accurate serum and plasma levels in picograms. Dallman *et al* demonstrated a relationship between IL-2 mRNA expression and acute rejection in fine-needle aspiration samples from renal allografts[43]. In general, however, studies of serum levels of a variety of cytokines have failed to distinguish reliably between rejection and infection.

1.5.4 Cytokines that mediate and regulate innate immunity

a) Type I interferons

There are three main types of interferon that are produced by human cells: interferon alpha, interferon beta, and. There is also an older system of nomenclature, that divides interferons into two types: type I interferons (interferon alpha and interferon beta) mainly produced by fibroblasts, epithelial cells and macrophages.

There are at least 25 sub-types of IFN-α. They are comprised of 166 amino acids and are mainly produced by leukocytes. Its receptor is a Type I multi-subunit receptor.

There is only one type of IFN-β which is contains166 glycosylated amino acids. It is mainly produced by fibroblasts, epithelial cells and macrophages. Interferon beta produced in mammalian cells is interferon beta-1a; interferon beta-1b is produced in bacteria.

b) Interferon gamma

The receptor for IFN-γ has two subunits, the ligand-binding chain (the α chain) and the signal-transducing chain (the β chain). As the ligand-binding (or α) chains interact with IFN-γ they dimerise and become associated with two signal transducing (or β) chains which leads to activation of the Janus kinases JAK1 and JAK2.

c) Tumour necrosis factor-α

Tumour necrosis factor**-**α (TNF- α), also known as cachectin, There are two TNF-receptors: TNF-RI (55kDa) and TNF-RII (75kDa), 19 ligands and 29 receptors that belong to the tumour-necrosis factor (TNF) superfamily have been identified.

d) Interleukin 1

Interleukin 1 (IL-1) is a general name for two proteins, IL-1a and IL-1b, the first of a small family of regulatory and inflammatory cytokines[44]. Along with IL-1 receptor antagonist (IL-1ra) and IL-18, these molecules play important roles in the up- and down-regulation of acute inflammation [45] .In the immune system, the production of IL-1 is induced, generally resulting in acute inflammation. IL-1b and TNF-a are generally thought of as prototypical pro-inflammatory cytokines. The effects of IL-1, however, are not limited to inflammation, as IL-1 has also been associated with bone formation and remodelling [46]insulin secretion[47] appetite regulation[48]fever induction[49]. IL-1 has also been known by a number of alternative names,

including lymphocyte activating factor, endogenous pyrogen, catabolin, hemopoietin-1, melanoma growth inhibition factor, and osteoclast activating factor.

Human IL-1a and IL-1b are synthesized as 31-33 kDa, variably glycosylated pro-cytokines. Although the pro-forms have no known biological activity, there may be a regulatory interaction between the pro- and mature segments both before and after cleavage. IL-1a and IL-1b exert their effects by binding to specific receptors. Two distinct IL-1 receptor binding proteins, plus a non-binding signalling accessory protein have been identified. Each have three extracellular immunoglobulin-like domains, qualifying them for membership to the type IV cytokine receptor family. IL-1 is expressed by many cells and has multiple functions including local inflammation.

e) Interleukin 6

Interleukin 6 (IL-6) may be considered the prototypic pleiotrophic cytokine. IL-6 induces cellular differentiation or expression of tissue-specific genes; it is involved in processes such as antibody production in B cells, acute-phase protein synthesis in hepatocytes, megakaryocyte maturation, cytotoxic T cell differentiation, and neural differentiation of PC12 (pheochromocytoma) cells. This is reflected in the variety of names originally assigned to IL-6, including Interferon b2, Hepatocyte Stimulating Factor, Cytotoxic T-cell Differentiation Factor, B cell Differentiation Factor (BCDF) and/or B cell Stimulatory Factor 2 (BSF2). Once all the activities associated with the various names for IL-6 became connected with one common gene, the name IL-6 was proposed for this molecule A number of cytokines make up an IL-6 cytokine family. Membership in this family is based on a helical cytokine structure consisting of two polypeptide chains, a ligand-binding chain (IL-6R) and a non-ligand-binding, signal-transducing chain (gp130)[50].

f) Interleukin 10

(CSIF/Cytokine Synthesis Inhibitory Factor)

All mature IL-10 family members have an α-helix common to their structure. IL-10 is involved in mast cell proliferation and mediates leukocyte secretion and chemotaxis[51]. On T cells, IL-10 inhibition of IFN-g production is mediated indirectly by accessory cells [52]. Additional effects on T cells, include: IL-10 induced CD8+ T cell chemotaxis[53], an inhibition of CD4+ T cell chemotaxis towards IL-8,[51] suppression of IL-2 production following activation[54], an inhibition of T cell apoptosis via Bcl-2 up-regulation,[55] and an interruption of T cell proliferation following low antigen exposure accompanied by B7/CD28 co-stimulation[56].

IL-10 has a number of related but distinct functions on B cells. In conjunction with TNF-β and CD40L, IL-10 induces IgA production in naïve (IgD+) B cells. It is believed that TGF-β/CD40L promotes class switching while IL-10 initiates differentiation and growth[57]. CD27/CD70 interaction in the presence of IL-10 promotes plasma cell formation from memory B cells[58].
On mast cells, IL-10 induces histamine release while blocking GM-CSF and TNF-α release. This effect may be autocrine as IL-10 is known to be released by mast cells in rat[59].In support of its pleiotrophic nature, IL-10 has the opposite effects on NK cells. Rather than blocking TNF-a and GM-CSF production, IL-10 promotes this function on NK cells[60]In addition, it potentiates IL-2 induced NK cell proliferation and facilitates IFN-γ secretion in NK cells primed by IL-18[61]In concert with both IL-12 and/or IL-18, IL-10 potentiates NK cell cytotoxicity[61].

IL-10 has a distinct anti-inflammatory impact on neutrophils. It inhibits the secretion of the chemokines MIP-1α, MIP-1β and IL-8[62]and blocks production of the pro-inflammatory mediators IL-1β and TNF-α[63]. In addition, it decreases the ability of neutrophils to produce superoxide, and as a result interferes with PMN mediated antibody-dependent cellular cytotoxicity[64].

On dendritic cells (DCs), IL-10 exhibits immunosuppressive effects. Macrophages, although phagocytic, are poor antigen-presenting cells and IL-10

seems to decrease the ability of DCs to stimulate T-cells (particularly Th1 type cells) [65]. Relative to MHC type II expression, it can be down-regulated[66], unchanged[67], or up-regulated[68]. At the regional level, IL-10 may block immunostimulation by inhibiting Langerhans cell migration in response to pro-inflammatory cytokines [69]. Alternatively, IL-10 blocks an inflammation-induced DC maturation, which results in a failure of DCs to migrate to regional nodes. The result is an immobile DC that will not stimulate T cells but will bind and therefore eliminate pro-inflammatory chemokines without responding to them[70]

IL-10 has a number of documented effects on monocytes. For example, IL-10 seems to reduce cell surface MHC type II expression[71] and also inhibits IL-12 production following stimulation. The connective tissue component Hyaluronectin, is now known to be secreted by monocytes in response to IL-10, which may have some importance in cell migration, where hyaluronectin is known to interrupt cell migration through the extracellular space[72].

g) Interleukin 12

IL-12, a cytokine produced by macrophages and B lymphocytes, has multiple effects on T-cells and NK cells, including stimulation of cytotoxic activity, proliferation, and promotion of Th1 development and IFN-gamma and TNF production [73, 74]The pleiotropic functions of IL-12 on T cells and NK cells include stimulation of cytotoxic activity, proliferation, and promotion of Th1 development[75].

h) Interleukin 15

Interleukin 15 (IL-15) is a novel cytokine that shares many biological properties with, but lacks amino acid sequence homology to, IL-2. IL-15 encodes a 162 amino acid precursor protein that is cleaved to generate the 114 aa residue mature IL-15. Although the structure of IL-15 has not been determined, it is predicted to be similar to IL-2 and other members of the four-helix bundle cytokine family[76].

High-affinity cell surface receptors for IL-15 have been detected on a variety of T cells and B cells, as well as non-lymphoid cells[77]. the beta and the gamma common chain subunits of the high-affinity IL-2 receptor complex are also required for IL-15 signal transduction and efficient internalization [77]. IL-15R alpha shares structural similarities with IL-2R alpha and Soluble IL-2R beta appears to bind human IL-15 with sufficiently high affinity making it an efficient IL-15 antagonist.

IL-15 mRNAs have been detected in a number of human tissues and cell types, including heart, lung, liver, placenta, skeletal muscle, adherent peripheral blood mononuclear cells, and epithelial and fibroblast cell lines. However, IL-15 mRNA is not detectable in activated peripheral blood T cells that contain high levels of IL-2 mRNA [77, 78]. Despite high levels of IL-15 mRNA being present in adherent peripheral blood mononuclear cells, IL-15 protein does not appear to be detectable in culture supernatant using a sensitive ELISA.

IL-15 has biological activities similar to IL-2 and has been shown to stimulate the growth of natural killer cells, activated peripheral blood T lymphocytes[77, 78], and B cells[79]. In addition, IL-15 has also been shown to be a chemo-attractant for human blood T lymphocytes [80] and to be able to induce lymphokine-activated killer (LAK) activity in NK cells.

By virtue of its activity as a stimulator of T cells, NK cells and LAK cells, IL-2 is currently in clinical trials testing its potential use in treatments for cancer and for viral infections. Because of its similar biological activities, IL-15 should have similar therapeutic potential.

1.5.5 Cytokines that mediate and regulate specific immunity

a) Interleukin 2

IL-2 is a monomeric, secreted glycoprotein with a molecular weight of 15 kDa. It exists in a globular structure with four α-helices folded in a configuration typical of the Type I cytokine family. IL-2 was discovered in 1975 as a growth

factor for bone marrow-derived T lymphocytes [1]. It was one of the first cytokines to be characterized at the molecular level. IL-2 exhibits a large number of pleiotropic effects on numerous target cells.

Cell type	Primary activities of IL-2
$CD4^+$ T cells	Induces expansion of antigen-specific clones via both proliferative and anti-apoptotic mechanisms. Augments production of other cytokines Required for differentiation to Th1 and Th2 subsets Induces apoptosis of activated T cells via Fas/FasL signalling (activation-induced cell death) Involved in development of $CD4^+CD25^+$ T regulatory cells (?)
$CD8^+$ T cells	Induces expansion of antigen-specific clones Augments cytokine secretion Augments cytolytic activity Induces proliferation of memory $CD8^+$ cells
B cells	Enhances antibody secretion Initiates immunoglobulin J chain transcription and synthesis Promotes proliferation
NK cells	Promotes proliferation Augments cytokine production Enhances cytolytic activity

Table 1 - Primary activities of IL-2

i. T-cell activation

IL-2 exerts its effects on many cell types, the most prominent of which is T-cell activation which rapidly leads to the de novo synthesis of IL-2. This is followed by expression of a high affinity IL-2 receptor permitting rapid and selective expansion of effector T cell populations activated by antigen[81]. A major function of IL-2 is to promote proliferation of both CD4+ and CD8+ T cells. IL-2-induced proliferation occurs via pro-proliferative signals through

the proto-oncogenes c-myc and c-fos, in combination with anti-apoptotic signals through Bcl-2 family members [82]. More recently, it has become clear that, in addition to anti-apoptotic signals, IL-2 also exerts effects on cellular metabolism and glycolysis that are necessary for long-term survival of T cells[83].

ii. Down-regulation of immune Reponses

Paradoxically, studies in IL-2 knockout mice have revealed that perhaps the most important activity of IL-2 is to down-regulate immune responses in order to prevent autoimmunity. These inhibitory effects of IL-2 create a negative feedback loop that is achieved by several mechanisms. Production of IL-2 is quite transient and so in the absence of continued antigenic stimulation, activated T cells die due to cytokine deprivation. Second, IL-2 initiates apoptosis through enhancing FasL expression on activated T cells. additionally there is good evidence that IL-2 may act during thymic development to prevent autoimmunity, probably by influencing the development of CD4+CD25+ T regulatory cells [84].

iii. Growth factor action

IL-2 is also a growth factor for natural killer (NK) cells together with IL-15, which signals through an essentially identical receptor [85]. IL-2 promotes production of NK-derived cytokines such as TNFα, IFNγ and GM-CSF. Furthermore, IL-12 and IL-2 act synergistically to enhance NK cytotoxic activity [86].

iv. Antibody production

In B cells IL- 2 has been associated with facilitating antibody secretion. In IgM-expressing B cells, IL-2 (along with IL-5) up-regulates expression of heavy and light chain genes as well as inducing de novo synthesis of the immunoglobulin J chain gene [87]. The latter is required for oligomerization of the IgM pentamer, and represents a tightly controlled stage in B cell activation [22]. As in T cells, IL-2 increases expression of IL-2Rα in B cells, enhancing their responsiveness to IL-2[88]].

Inhibition of IL 2

Calcineurin is the target of several potent immunosuppressive drugs (including cyclosporin A and rapamycin), which suppress T cell activity by inhibiting IL-2 secretion.

The majority of IL-2 is derived from activated CD4+ T cells. Whereas most or all T cells produce IL-2 immediately following antigen stimulation, only the Th1 subset produces it in large amounts after T helper cell differentiation [43]. In addition, CD8+ T cells also secrete substantial quantities of IL-2 after stimulation of their T cell receptors.

A number of drugs act at various points in the TCR signalling pathway to block IL-2 production. For example, cyclosporin A (CsA) is a cyclic oligopeptide and a potent immunosuppressant that blocks the activity of calcineurin, and thus prevents NFAT from gaining access to the nucleus. Rapamycin and FK506, other common immunosuppressants in clinical use, also block calcineurin, although by a different mechanism [54], [55] and [56].

IL-2 is crucial for the maintenance of immune homeostasis. First, IL-2 is important for the clonal expansion of most types of activated T cells. IL-2 is vital for determining the magnitude and duration of primary and memory immune responses. Second, IL-2 plays a central role in down-regulating immune responses. Its absence results in severe autoimmunity due to a failure to eliminate activated T cells [89]. Third, IL-2 opposes IL-15 in maintaining CD8+ T cell memory responses [90]. Finally, recent studies have indicated that a major function of the IL-2/IL-2 receptor system lies in directing development and function of T regulatory cells [91].

A number of agents Cyclosporin A, tacrolimus (FK506) and sirolimus (rapamycin) target IL-2 production and/or the IL-2 signalling cascade. Anti-IL-2Rα antibodies are also currently used in anti-rejection regimens for kidney transplantation, by specifically blocking IL-2-mediated signalling through high affinity receptors[92].

b) Interleukin 4-see IL-13

c) Interleukin 5

Human IL-5 is a 134 amino acid (aa) polypeptide with a predicted mass of 12.5 kDa Although many cells contribute to the general process known as inflammation, eosinophils are noted for their contribution to late phase allergic-type disorders. Interleukin 5 (IL-5), is a key player in the co-ordination and orchestration of eosinophil-based inflammatory processes[93].

d) Interleukin 13

Interleukin 13 (IL-13) is closely related to interleukin 4 (IL-4). They display overlapping functions, and the genes for the human proteins are both found on chromosome 5q. IL-13 is produced by activated Th0, Th1-like cells, Th2-like cells and CD8-positive T cells. IL-13 has multiple effects on the differentiation and functions of monocytes/macrophages. It can suppress the cytotoxic functions of monocytes/macrophages, the production of pro-inflammatory cytokines, and up-regulate the production of IL-1rα. The similarities and overlapping functions of IL-4 and IL-13 led to interest in the components of the IL-13 receptor (IL-13R) complex and the relationship of IL-13R to the IL-4 receptor (IL-4R). The IL-4Rα chain plays an important role in IL-13 signalling, despite it having a low affinity for IL-13[94].

e) Interleukin 16

Interleukin 16 (IL-16) is a pro-inflammatory cytokine that is chemotactic for CD4+ T lymphocytes, monocytes and eosinophils. In addition to inducing chemotaxis, IL-16 can up-regulate IL-2 receptor[95] and HLA-DR4 expression and inhibit T cell receptor (TcR)/CD3-dependent activation[96]. 7 IL-16 is a unique cytokine with no significant sequence homology to other well-characterized cytokines or chemokines. IL-16 was originally identified as a homotetramer consisting of individual 14 kDa monomers of 130 amino acids (aa) each. The gene for IL-16 maps to chromosome 15.

CD4 serves as a signal-transducing receptor for IL-16. Expression of CD4 is required for mediating IL-16 functions[96]. Interaction between IL-16 and CD4 can specifically initiate an increase in intracytoplasmic calcium and inositol trisphosphate and translocation of protein kinase C from the cytosol to the cell membrane. IL-16-induced functions can be inhibited by a monomeric Fab of an anti-CD4 (OKT4) monoclonal antibody[97].

Sources of IL-16 include epithelial cells, mast cells, lymphocytes, macrophages, synovial fibroblasts, and eosinophils. IL-16 mRNA is constitutively expressed in both CD4+ and CD8+ cells, however, synthesis is induced in T lymphocytes upon exposure to antigen. IL-16 may also be secreted by activated CD8+ cells in response to histamine or serotonin.

f) Interleukin 17

IL-17 (also known as CTLA-8 (cytotoxic T cell lymphocyte-associated antigen)) is a variably glycosylated, 20-30 kDa homodimeric polypeptide reportedly secreted by $CD4^+$ activated memory ($CD45^+RO^+$) T cells.[5, 14] The IL-17 gene codes for a 155 amino acid residue protein that consists of a 19 aa residue signal sequence and a 136 aa residue mature segment.

It is suggested that IL-17 may be a major vehicle by which T cells communicate with the haematopoietic system. In particular, fibroblasts, when cultured in the presence of IL-17, are able to sustain $CD34^+$ haematopoietic progenitor cells and direct their maturation towards neutrophils[98], IL-17 has been demonstrated to induce IL-6, IL-8 and G-CSF production by fibroblasts (and endothelium), and these three cytokines are known to impact haematopoiesis[99].

g) Interferon-γ

IFN-gamma, a 34 kDa homo-dimeric glycoprotein, is essentially restricted to activated CD4+ Th1 T cells, CD8+ T-T cells, and NK cells[100]. For each cell

type, IFN-gamma secretion is further restricted by the availability of IFN-gamma-inducing cytokines such as IL-12 and TNF-alpha, which arise from accessory cells following activation[100]. One of the most important consequences of IFN-gamma secretion is the activation of macrophages. This is achieved through the induction of reactive oxygen intermediates and nitrogen monoxide (NO), which activate a variety of anti-bacterial, anti-tumour and anti-viral responses[100]. In addition, IFN-gamma contributes to endothelial cell activation, Th1 cell development, and up-regulation of MHC expression on both professional APCs and non-APCs. This makes the regulation of IFN-gamma an extremely important step in the overall scheme of an inflammatory response[100].

h) TGF-beta

Transforming growth factor beta (TGF-beta) is a stable, multifunctional polypeptide growth factor. Specific receptors for this protein have been found on almost all mammalian cell types. The function of the molecule varies depending on the cell type and growth conditions. Generally, TGF-beta is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin. TGF-beta, (known as TGF-beta1) is only one of a family of regulatory proteins consisting of a number of proteins distantly related to TGF-beta 1 (30 - 40% sequence homology) and a number of more closely related proteins (70 - 80% sequence homology).

TGF-beta1 is thought to be important in the pathogenesis of chronic allograft nephropathy. Although the cause of CAN is not fully understood, TGF-beta1 may be one important since this cytokine promotes fibrosis by stimulating the production of extracellular matrix components, such as collagen and fibronectin. Sharma *et al* found a significant correlation between TGF-beta1 mRNA levels and renal allograft interstitial fibrosis and chronic allograft nephropathy [101]. Low TGF-beta production in donor-recipient combinations mismatched for TGF-beta genotype has been associated with risk for early rejection and poorer graft function at 4 years [102]. Ochsner *et al* postulate that

tailoring immunosuppressive therapy according to the TGF-beta1 genotype might result in improved long-term function [103].

1.6 Historical overview of the development of immunosuppresion

1.6.1 The First Kidney Transplant.

In 1954, the first successful kidney transplant between identical twins was performed by Joseph E. Murray and J Hartwell in the Peter Bent Brigham Hospital in Boston, USA[104]. The first successful kidney transplant in UK was performed in Edinburgh by Sir Michael Woodruff and his team on 30th October, 1960. However, the early years were known as “the black years” due to the consistently high graft failure and patient mortality rates [105] .

1.6.2 Strategies to overcome the immune system.

The biological response to transplanted organs involves both the major histocompatibility (MHC) complex, which modulates rejection, and variable expression of chemokines and cytokines in the recipient. The overall interaction ultimately determines the type and degree of immune responsiveness to the foreign tissue. There have been various attempts to suppress the immune response which have over time become more targeted.

1.6.2.1 Total body irradiation

The initial successes were in identical twins, overcoming non-identical HLA mismatch problems and their inevitable immune responses. In order to overcome the genetic mismatch it was soon realised that the recipients’ immune system must be overcome if a kidney allograft were to have meaningful function. From 1956 total body irradiation followed by bone marrow transplant was employed to reduce graft rejection. However, irradiation could lead to destruction of the nervous system, fluid loss and bacterial invasion, and could cause death within a few hours to days. Despite occasional successes the irradiated recipients suffered from unacceptably high

mortality rates. This situation obviated the need for more specific and controllable methods of immunosuppression.

1.6.2.2 Generalised Immunosuppressive agents

In 1959, two Boston haematologists, Robert Schwartz and William Dameshek reported that the activity of adult rabbits to beef protein could be suppressed by the anticancer drug 6-mercaptopurine. By inhibiting RNA and DNA synthesis it diminished maturation and proliferation of all rapidly dividing cells including lymphoid cells. When antigen and drug were administered together, the rabbits remained specifically non-reactive to the foreign protein even after cessation of the agent but still responded to third party protein. This state was described as "drug induced immunological tolerance" [105] .

1.6.2.3 Specific Immunosuppressive agents

I. Azathioprine and Corticosteroids

In 1961, Calne, Peart and Porter launched a transplantation programme using azathioprine and corticosteroids. In April 1962, R.Y. Calne and J.E. Murray reported the successful use of azathioprine, on a kidney transplant patient in Boston, USA. The second half of this first batch of azathioprine was used in a successful renal transplantation in Edinburgh in the same year. This important development - maintenance immunosuppression with azathioprine and steroids in combination with the treatment of rejection episodes with high doses of steroids - remained the mainstay of clinical immunosuppressive regimes for the next two decades.

II. Azathioprine alone

A further clinically relevant advance came from Thomas Starzl and Thomas Marchioro, who presented their results with twenty-seven renal transplants performed over the previous ten months[106] - twenty-five from living donors, both related and unrelated. Starzl and Marchioro used azathioprine alone as primary immunosuppression, but reversed the virtually inevitable acute rejection episode in over 90 per cent of the cases with high doses of prednisolone, and actinomycin C, an antibiotic that killed populations of white

blood cells. Eighteen of their patients (67%) remained alive with satisfactory graft function.

Of the two hundred and sixteen recipients of kidney allografts performed up to 1963 in North America and Europe 52% of those receiving allografts from related donors and 81% of those receiving allografts from non-related donors had died. Only 4% of all allografts functioned for more than a year. In contrast 76% of identical twin (or irradiated non-identical twin) recipients were still alive. This indicated that kidney transplantation was still experimental and not yet a therapeutic procedure.

III. Anti-lymphocyte Serum / Anti-Lymphocyte Globulin

In parallel to the above developments, research in immunology and cell biology was creating an alternate approach. In 1951, Sir Michael Woodruff published a paper on his work on anti-lymphocytic serum (ALS) in the rat[107]. He modified the technique used by Cruickshank in 1941 and produced rabbit-anti-rat ALS induce lymphopaenia. This was a more specific biological method to suppress the immune system by acting directly on the lymphocytes. Work continued throughout the 1960s, and Woodruff found that lymph drainage and administration of ALS resulted in prolonged homograft survival in rats, where the maximum effect was achieved with treatment both before and after graft transplantation[108]. In 1966, Sir Michael Woodruff and colleagues showed that ALS against dog thoracic lymphocytes prolonged the survival of whole kidney homotransplants in dogs. Sir Michael Woodruff and his team made anti-lymphocyte globulins (ALG) and ALS in collaboration with colleagues at the Royal School of Veterinary Science in Edinburgh. Theses polyclonal antibodies were made in horses.

Understanding of ALG was important if it were later to be used clinically in humans, and Keith James and Sir Michael Woodruff undertook extensive research to establish this. The team investigated the mode of action of ALG, the kinds of immune response which it suppressed, and the nature of the molecular characteristics of the antibody. ALG was found to be predominantly

of the IgG class. Furthermore, intact IgG was required to give a biological effect. Antibody fragments, e.g. Fab, did not work since they did not fix complement, and could not lead to complement-mediated lysis of lymphocytes (James K, 1967). ALG also suppressed antibody formation experimentally [109]. Suppression was dependent on the antigen, species of rat, and the timing of antigen challenge (ALG was injected before antigen challenge to give immunosuppression). ALG was later used in humans as an adjunct to other immunosuppressants, and it was observed that grafts were functioning after 5 years in 4 out of 14 renal transplant patients who received ALG in addition to azathioprine and prednisolone [110].

By 1972 advances were obvious, particularly with kidneys from living related sources; the rate of graft survival had improved to 75 percent at one year. Results with cadaver organs increased from 25 percent to 45 percent, a relatively mediocre figure that was to remain static over the next decade. A few long-surviving patients had returned to normal lives.

IV. Blood Transfusion and Low-dose steroids

Terasaki demonstrated that long-term prognosis in humans post-transplant was directly related to the number of pre-transplant transfusions [111]. It was thought that blood transfusion enhanced graft survival. In the process, only frozen washed red cells were used (leukocyte-depleted). Later on, there was more evidence that blood transfusion enhanced graft survival, possibly by inducing tolerance. After a while, it became usual practice in Edinburgh to transfuse 4 units of blood before transplant. It was shown that blood transfusion together with a low-dose steroid regime increased the 3-year graft survival rate from less than 40% to 66% [112]. Reductions in acute rejection reduced the numerous side effects associated with high steroid intake, including mortality and bone morbidity. However, transfusion was not without problems, some patients became highly sensitised to transplant antigens, making transplantation very difficult. With the discovery of more effective anti-rejection agents, pre-transplant transfusions were abandoned.

With slowly accumulating clinical successes and intriguing scientific findings, a series of new strategies were initiated in hope of improving the results further. Management of end-stage renal disease became better understood. Tissue matching, with its potential of offering the best organ to the best recipient, appeared full of promise, particularly as hope increased that the dosages and toxicities of the existing immunosuppressive modalities could be reduced. New methods of preservation and storage allowed more time to transfer a kidney to a well-matched recipient. More effective dialysis techniques sustained more patients as they awaited transplantation. More institutions initiated transplant programs. Progressive understanding of the biology of the host immune responses, and the realization that a variety of chemical and biological agents could modulate their activity and increase the survival of foreign organs, focused investigative efforts.

V. Cyclosporin A

In 1969 - 1970, two new strains of fungi were isolated. Only one of the strains, *Tolypocladium inflatium (Gans)*, could be grown in culture and a fungal product was isolated from the culture broth. The extract, eventually characterized chemically as a ring of eleven protein fragments, became available for biological screening. Initial analysis was unremarkable until Jean Borel, examined the materials pharmacological properties. He quickly discovered that one of the metabolites isolated from the extract was markedly immunosuppressive. Soon the influence of this factor, Cyclosporin A, on both cellular and humoral immune activity had been established in cultured cells. The new agent reversibly inhibited the function of T lymphocytes and was nontoxic to several cell populations in a variety of animal species. The selective interactions of Cyclosporin A with the host immune responses contrasted to those other immunosuppressants available, which indiscriminately destroyed rapidly dividing cells throughout the body.

In November 1978 the Lancet published two short papers that radically changed the nature of clinical transplantation. In the first, a group in London showed that treatment with a new immunosuppressive drug, Cyclosporin A,

substantially diminished the incidence and severity of GVHD in bone marrow transplant recipients [113]. In the second, Roy Calne and his Cambridge associates reported that the same agent had been strikingly effective in several patients with kidney grafts [114].

One year later they described in detail their results with thirty-four recipients of thirty-six organ allografts, all treated with Cyclosporin A [115]. Twenty-six of the thirty-two kidneys continued to function, as did two pancreases and two livers. Twenty patients received no adjunctive steroids. No additional chemical agent such as azathioprine was used in fifteen individuals. Compared to the clinical results of the previous fifteen years, these data were unmatched.

In 1982 Calne and White reported one-year actuarial survival of 82 percent of sixty cadaver- donor kidney grafts in fifty-nine patients, a figure far superior to results ever achieved using older forms of immunosuppression[116].
Two international clinical controlled trials began to enrol patients. By 1983, overall results from both multicenter trials and from individual units, had shown an approximately 20 percent increase in one-year graft function compared to conventional therapy, and a 10-15 graft survival percent advantage at three and five years. Liver and heart transplantation also improved substantially.

Cyclosporin dramatically increased the number of organs grafted and numbers of persons demanding them. In 1976, worldwide, six scientific papers on the subject had been published; in 1980 there were 107, and within a few years, there were thousands.

Within a few years of the advent of Cyclosporin, additional agents were introduced with ever-increasing frequency. By the end of the 1990s several clinical trials were nearing completion and new agents became generally available [117]. Improving success was paramount for recipient and physician when faced with a rejection episode unresponsive to standard therapy and the threat of graft loss, particularly as the relatively low number of available

organs often meant years of waiting for a new one. The possibility of more effective drugs encouraged clinicians interested in improving results and also basic scientists who used the compounds as probes to examine the complexities of molecular events occurring in activated cells. The changing face of the increasingly powerful and competitive pharmaceutical industry hastened the development of the new drugs. As the breadth of the field increased and immunosuppression was discovered to be financially lucrative, the industrialization phase of transplantation commenced.

Three new agents in particular, mycophenolate mofetil (CellCept), tacrolimus (Prograf), and rapamycin (Sirolimus) used alone or in combination with Cyclosporin, have improved the short-term results of organ transplantation.

VI. Mycophenolate mofetil

Mycophenolate mofetil was shown to reduce the incidence of acute rejection episodes in the short term compared to the use of azathioprine [118]. In the longer term when the diagnosis of chronic rejection was made and patients were converted from cyclosporin to mycophenolate better graft function with lower rates of graft loss was seen [119]. In an animal model mycophenolate was shown to reduce intimal hyperplasia - one of the pathological processes that leads to chronic allograft nephropathy [120]. Mycophenolate mofetil was shown to improve long-term graft survival following renal transplantation in patients with delayed graft function [121].

VII. Tacrolimus

Hariharan *et al* converted kidney/pancreas transplant patients from cyclosporin to tacrolimus. Renal and pancreatic function remained stable during long-term follow-up and with a tendency toward a lower rejection rate [122, 123].

A phase III U.S. Multicenter Trial compared tacrolimus - and Cyclosporin (CsA)-based immunosuppressive therapy. A significant reduction in the incidence and severity of acute rejection episodes was found among patients maintained on tacrolimus [124]. Where there had been a progressive decrease in renal function conversion from Cyclosporin to tacrolimus was found to

stabilize or improve graft function [125], and was highly effective as monotherapy (i.e. steroid free) over a ten year period [126].

VIII. Sirolimus

Two studies looking at the use of sirolimus monotherapy in order to reduce nephrotoxicity from the chronic use of calcineurin inhibitors found a significant reduction in histological damage and superior renal function in the group maintained on sirolimus alone [127, 128]. Sirolimus has also been used to prevent further deterioration in renal function caused by calcineurin inhibitor induced chronic allograft nephropathy[129, 130].

Following the discovery of Tacrolimus, isolated from the bacterium Streptomyces tsukubaensis [131], Calne and Starzl were in the audience during a presentation at the Transplantation Society Congress in 1986, on the immunosuppressive effectiveness of Tacrolimus in a rat heart-graft model. Calne had already tested the agent and felt that it was exceptionally toxic to the gastrointestinal tract of dogs and produced significant inflammation of small blood vessels. He also noted that it was considerably more potent than cyclosporin. Starzl, in contrast, was so enthused by the data that he promptly travelled to Japan, to determine that organ allograft survival was prolonged in treated rat, dog, monkey, and baboon recipients

The biochemical structure of the new agent was shown to be distinct from that of cyclosporin, it bound to related but not similar protein receptors in the cell and interfered early in the cell activation process. Thus, it blocked generation of various lymphocyte products that mediate rejection. Starzl took on the drug and controlled its use for months. Having rescued it from virtual oblivion following the initial adverse reports of its toxicity, he persuaded the Food and Drug Administration (FDA) to allow him to use Tacrolimus in selected patients. He soon reported that the agent was strikingly effective in human recipients of liver, small bowel, heart, pancreas, and kidneys - perhaps most obviously in its ability to rescue liver grafts failing from rejection episodes resistant to steroids or to anti T-cell monoclonal antibodies [132].

As others began to use FK, and data from two large multicenter trials eventually became available, the agent was increasingly accepted. One year actuarial graft survival differed little from that of Cyclosporin treated hosts and acute rejection could often be successfully reversed by conversion from Cyclosporin to FK. Side effects, were significant, FK506 was found to be extremely toxic to kidneys, causing some heart, liver, and multi organ recipients to require dialysis. Up to 20 percent of liver recipients developed mild central nervous system abnormalities. The onset of diabetes was an unexpected problem, with one third of renal transplant patients in a large study requiring insulin treatment [133]. Despite these adverse reactions, the use of FK has gradually has become more routine, especially in multiorgan transplantation.

Development and use of Immunosuppression in Kidney Transplantation	
1954	First successful identical-twin kidney transplant
1961	Kidney transplants in irradiated hosts, unacceptably high mortality rates
1960 - 1963	Clinical use of chemical immunosuppression (azathioprine).
1963	52% of those receiving allografts from related donors and 81% of those receiving allografts from non-related donors had died
1964	Addition of steroids to reverse acute rejection. Azathioprine, steroids in combination mainstay of clinical immunosuppressive regimes for the next two decades.
1972	75 percent graft survival at one year in recipients of kidneys from living related donors. One year graft survival of deceased donor kidneys remained at 25 percent to 45 percent next decade.
1979	Twenty-six of the thirty-two kidneys survived in one study where patients received cyclosporin as baseline immunosuppression[23].
1982	82 percent 1 and 2 year graft survival.[24]
1986	Introduction of FK (tacrolimus)
1988 – 1996	One-year survival rate for grafts from living donors increased from 88.8 to 93.9 percent, and the rate for deceased grafts increased from 75.7 to 87.7 percent.[32]
1995	Clinical introduction of mycophenolate mofetil
1999	Rapamycin (sirolimus) trials
2003	1 year graft survival; 89.2% (deceased donor) and 94.3% (living donor). OPTN/SRTR Data as of August 1, 2003.

Table 2 - Timeline; Development and use of Immunosuppression in Kidney Transplantation

1.7 Immunosuppression

Mechanism of Action.

During the past 50 years, many immunosuppressive drugs have been discovered empirically. Often their mechanisms of action were established long after their discovery. Eventually these mechanisms were found to fall into five groups:

I. Regulators of gene expression;
II. Alkylating agents;
III. Inhibitors of de novo purine and pyrimidine synthesis;
IV. Inhibitors of kinases and phosphatases.
V. Targeted immunosuppression - Monoclonal Antibodies

1.7.1 Regulators of gene expression

Corticosteroid is a glucocorticoid-based medication that works principally to block T cell and APC derived cytokine and cytokine-receptor expression. The major elements blocked are IL-1 and IL-6. Secondary effects of corticosteroids include the blocking of IL-2, INF-gamma, and TNF-alpha. These elements, notably IL-1, are essential for lymphocyte-APC communication. A decrease in production effectively obstructs an APC's capacity to activate allograft-specific lymphocytes. As a result, threat of acute rejection is reduced. Corticosteroids have a hydrophobic structure that allows them to easily diffuse into cells and bind to specific cytoplasmic receptors. The resulting complexes progress to the nucleus, where they are able to inhibit the transcription of the genes of the cytokines named above.

Corticosteroids are used in maintenance immunosuppression, and the treatment of acute rejection. Oral, as well as intravenous routes are commonly used. A high dose of intravenous methylprednisolone is usually given immediately before and during the transplantation procedure. Methylprednisolone or prednisone is often continued post-operatively for several days at high doses, and is then tapered to a maintenance dose. The maintenance dose consists of orally administered prednisone, and is normally 5-10 mg/day (0.1mg/kg). Side

effects include: hypertension, hyperlipidaemia, osteoporosis, weight gain, cushingoid appearance, opportunistic infection, glaucoma, ulcer formation, hyperglycemia - progressing to steroid induced diabetes.

1.7.2 Alkylating agents

Alkylating agents such as cyclophosphamide are organic chemicals that transfer alkyl groups to other molecules. Alkylation of DNA bases preferentially suppresses immune responses mediated by B-lymphocytes. Alkylating agents can cause pancytopaenia and haemorrhagic cystitis. They are also mutagenic and can increase the risk of developing cancer and are not used for the purposes of Immunosuppression in transplantation [134]

1.7.3 Inhibitors of de novo purine and pyrimidine synthesis

a) Azathioprine (Imuran®)

Azathioprine is hydrolyzed in the blood to 6-mercaptopurine. In this form as a purine analogue and antimetabolite, it is incorporated into DNA, inhibiting nucleotide synthesis by causing feedback inhibition in the early stages of purine metabolism. This prevents mitosis and proliferation of rapidly dividing cells, such as activated B and T lymphocytes. Through this action, Azathioprine is able to block most T-cell functions and inhibit primary antibody synthesis. Azathioprine has little effect on established immune responses, and is therefore effective in the prevention and not the treatment of acute rejection. Side effects include: bone marrow depletion/suppression, thrombocytopenia, anaemia, pancreatitis, hepatotoxicity, and neoplasia [134].

b) Mycophenolate Mofetil (CellCept®)

Mycophenolate Mofetil (MMF) is and rapidly hydrolyzed in the blood to its active form mycophenolic acid (MPA). Mycophenolic acid inhibits the enzyme inosine monophsphate dehydrogenase (IMPDH), in the de novo pathway of guanosine nucleotide synthesis. Rapidly dividing cells, such as activated lymphocytes, depend on the de novo pathway for the production of purines necessary for RNA and DNA synthesis. In this way, activated lymphocytes are selectively inhibited, their proliferative responses slowed and apoptosis of activated T-lymphocytes induced.

The unique property of MMF is its lack of atherogenic and chronic nephrotoxic adverse effects. Adverse effects are primarily gastrointestinal (e.g., nausea and/or vomiting, diarrhoea, gastritis, duodenitis, oesophagitis, ulcers). Other adverse events were related to bone marrow suppression (e.g., leukopaenia, anaemia, and thrombocytopenia). MMF is normally taken orally, 0.5-1.0 g twice daily. Side effects include: leukopaenia, thrombocytopenia, nausea, opportunistic infection, malignancies, and gastrointestinal upset [134].

1.7.4 Inhibitors of kinases and phosphatases.

The common mechanisms of calcineurin inhibitors converge at the inhibition of the calcineurin. This inhibition ultimately inhibits the production and secretion of IL-2. The interaction between IL-2 and the IL-2 receptor is crucial in the activation and differentiation of B and T cells. Therefore, halting the rejection process at this step is highly effective at fighting rejection.

a) Cyclosporin

Cyclosporin is a small fungal cyclic peptide that binds to cyclophilin and complexes with calcineurin. This inhibits phosphorylation of nuclear regulatory proteins which suppresses the activation of critical cytokine genes that promote T-cell activation (Il-2, Il-4, IFNγ, TNF-α). Calcineurin inhibitors are used as long-term or maintenance immunosuppression. Cyclosporin is available as an emulsion or micro-emulsions. Maintenance doses are administered orally typically 5-10 mg/kg/day being given in two doses. This is usually taken along with an antiproliferative agent and prednisone. Side effects include nephrotoxicity (partially reversible), hypertension, tremor, coronary artery disease, hirsutism, gingival hyperplasia, opportunistic infections malignancies, hyperuricaemia, hepatotoxicity, and hypertrichosis [134].

b) Tacrolimus

Tacrolimus is a macrolide antibiotic which binds to the cytosolic protein FKPB-121. This complex inhibits calcineurin in a similar manner to cyclosporin. Tacrolimus is available in oral tablets and as an intravenous formulation. A maintenance dose is usually 0.15 mg/kg/day given in two doses. Side effects include nephrotoxicity (partially reversible), hypertension, hyperkalaemia, hypomagnesaemia, alopecia, hyperglycemia (diabetogenic

potential stronger than cyclosporin), opportunistic infections, and malignancies [134].

c) Sirolimus (Rapamune®)

Sirolimus is a highly potent macrolide antibiotic that has a similar chemical structure to tacrolimus. Sirolimus binds to the same protein as Tacrolimus: FKBP-12. Instead of inhibiting calcineurin as tacrolimus does, this complex inhibits mTOR (mammalian target of rapamycin). This inhibition prevents the progression of T cells late in the cell cycle from the G1 to the S phase of the cell cycle by blocking signalling downstream of the IL-2 receptor. Therefore it is able to block delayed type hypersensitivity (DTH) reactions, cytotoxic t-cell activity, and humoral responses directed against a transplanted organ. Rapamycin inhibits kinases required for cell cycling and responses to IL-2 and also induces apoptosis of activated T-lymphocytes. Sirolimus is taken orally, at a dose of 2-5 mg once a day. Side effects include hypercholesterolaemia, hypertriglyceridaemia, leukopaenia and thrombocytopaenia [134].

1.7.5 Targeted immunosuppression - Monoclonal Antibodies

Immunosuppression can be directed to block the action of cytokines in a specific way. Anti IL-2 receptor (CD25) monoclonal antibodies against the p55 chain of the receptor (only expressed by activated and not resting T-cells) have been used in clinical trials in combination with conventional immunosuppression with encouraging results. Initially, the antibodies used were mouse derived which has the disadvantage of causing a strong anti immunoglobulin response by the patient, diminishing the effectiveness of the treatment. However, these have now been ‘humanised’ by creating a chimeric antibody with a mouse Fab fragment, and a human Fc portion. This has significantly reduced the immunogenicity of the molecules. The impact in transplantation has been the use of monoclonal antibodies in “high risk” recipients - those with a high panel reactive antibody, those with a minor degree of positive cross-match (e.g. to b-lymphocytes) and even those with ABO incompatibility [10]. Prior to their use transplantation would not have been contemplated.

1.8 Summary

In Summary, over the last 50 years the outcome of kidney transplantation has greatly improved. Improved graft survival is attributed to; advances in immunosuppression, better methods of cytotoxic antibody detection and human lymphocyte antigen matching. However, as we can see if we compare outcomes as described by Hariharan *et al* and those described by The U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients we can see that the rate of improvement is dramatically slowing and reaching its plateau.(See figure 4).

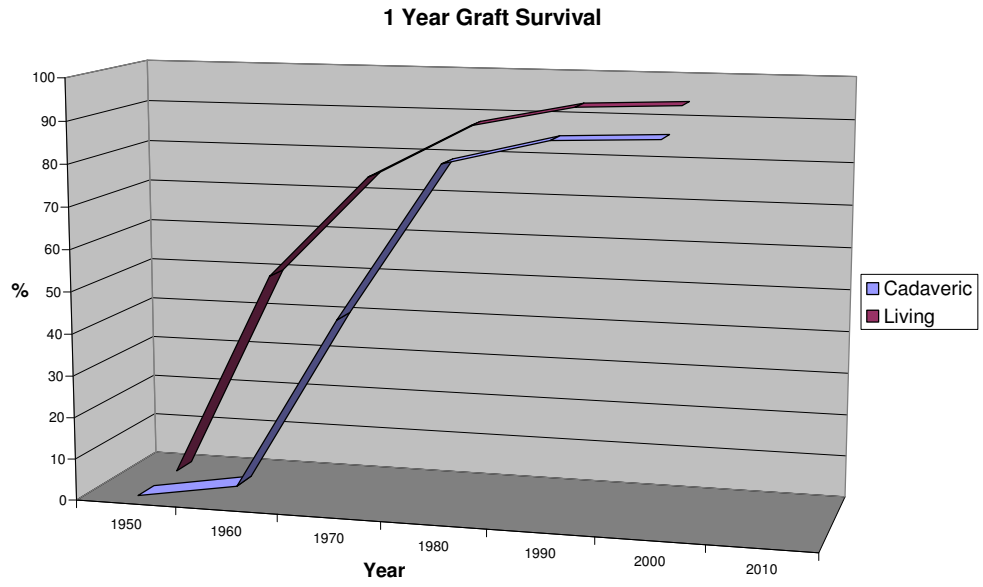

Figure 4 - U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: 1 Year Graft Survival

It seems to be the case that all known correctable and optimiseable factors have been taken into account and the optimum graft outcome has been achieved. The purpose of the thesis is to identify potential biomarkers and explore unknown factors which interplay between the graft and the host in an attempt to explain the overall graft attrition rate which remains at 6.4% per year for deceased donor and 4.5% per year for living donor kidneys averaged over a 10 year period.

1.9 History of Microarray

i. Array substrates

Traditional hybridisation assays were developed in the 1970's. They used flexible membranes as the substrate such as nitrocellulose and nylon. These early filter based "macroarrays" only included a limited number of targeted genes (1980's) and constant effort was made to miniaturize the procedure to increase the numbers of genes made available for analysis and reduce the sample requirement, resulting in the microarray in the 1990's (see. Figure 5)

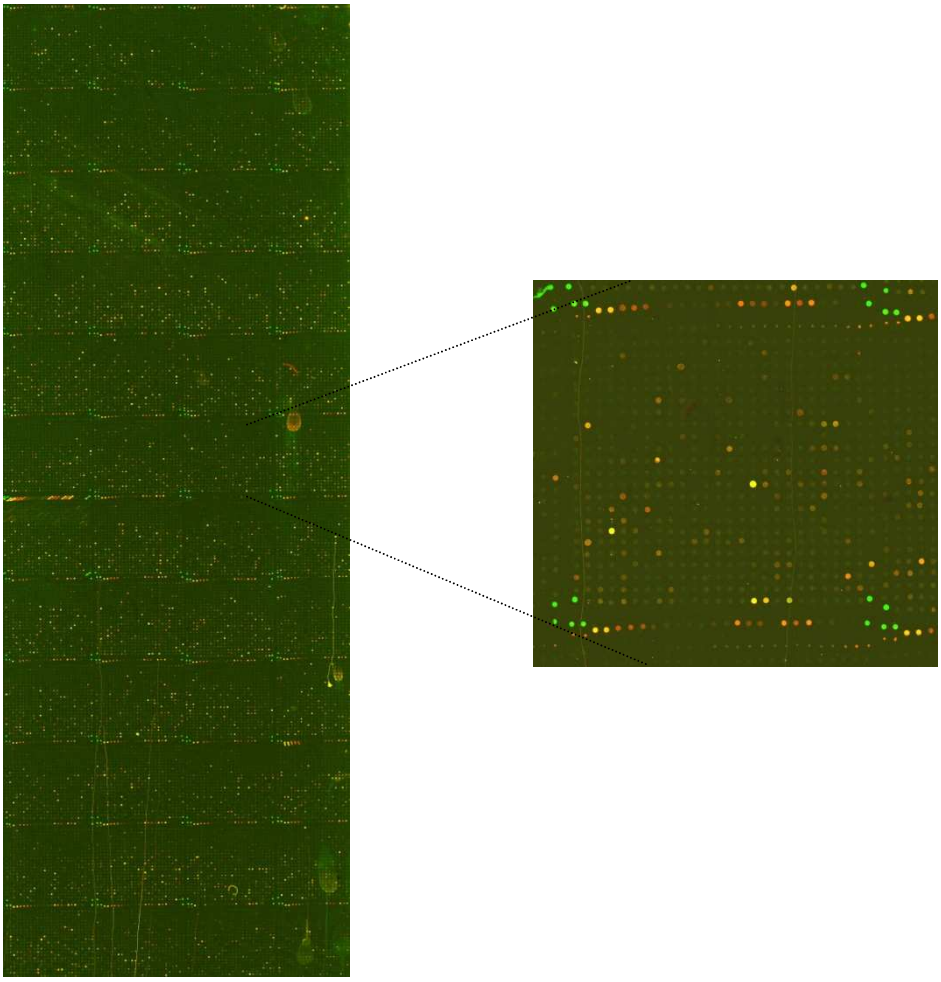

Figure 5 - Image of microarray slide following hybridisation .It was taken from experiment 4 produced, by Genepix Pro 6.0

ii. The microarray slide

In contrast to the filter based array the microarray utilized solid media (glass), which enables the deposition of small amounts of biochemical material to be placed in a precisely defined position. The non-porous surface permits the use of small sample volumes, enabling high sample concentrations and rapid hybridisation kinetics. The solid substrate also allows uniform surface for attachment of the array elements. The inherent flatness and uniformity allows parallelism, which provides a significant increase in the accuracy of array data. Each spot represents a high-density array of oligo-nucleotides adherent to the slide surface. The immobilized DNA selectively retrieves genes or sequences of interest when the array is hybridised to a mixture of complimentary sequences (the probe). The rate of hybridisation is related to concentration of the probe and is therefore proportional to the relative abundance of mRNA in the hybridising sample[135].

1.10 Biomarkers

Recently attention has been directed to cytokines / chemokines in the transplanted organs at time of transplantation. The question that has been asked is whether specific cytokines /chemokines enhance or decrease this immune response.

This may explain why some patients, despite receiving the same mismatched Major Histocompatibility Complex (MHC) alleles and the same immunosuppressive drugs have more rejection than others. Studies of these biomarkers within organ transplants have been hampered by the lack of a technique, which allows us to examine large numbers of these markers using only small amounts of tissue. This situation has changed recently with the introduction of the microarray technique, which has been shown to be useful in screening gene expression in kidney transplant biopsies [136, 137]. The emphasis from a traditional gene-by-gene approach has switched to looking at complete sets of genes. The new emphasis on the complete picture rather than on its component parts is driven by the completion of the human genome sequencing project [138]and the advances in microarray technologies that transform these advances into powerful research tools.

Gene Expression Profiling

At the RNA level, gene expression profiling gives us a snapshot of all the RNA transcripts that are expressed at a time zero (pre-transplantation) if we can be confident that mRNA levels in a renal biopsy are representative of those in the entire kidney [2]. Further snapshots can be taken which are temporally related to the first (e.g. 6 weeks later during acute rejection and subsequently at times of graft dysfunction). Spatial relationships can be examined (e.g. comparing snapshots in a number of grafts which undergo similar intra-graft events). Further to this each snapshot can be correlated to donor and recipient characteristics, time of biopsy, severity of rejection, immunosuppressant and many other variables. Bioinformatics tools used to analyse these large data file prove to be useful in identifying new biomarkers, or can confirm the results of other experiments using ELISA or PCR.

Tumour necrosis factor α (TNF- α) is an examples of a cytokines with relevant to transplantation. Another, interleukin 10 (IL-10) has been associated with the occurrence of acute rejection [3]. Further microarray findings support the positive role of TGF-β, as an anti-inflammatory cytokine [4, 5] but more specifically it has been shown to inhibit B cells, T helper cells, cytotoxic T cells and macrophages [6, 7].Border *et al* suggest that the overproduction of or prolonged exposure to TGF-β leads to excessive deposition of extra-cellular membrane components, eventually leading to progressive renal scarring [8, 9]. It has also been shown to have a role in the regulation of extra-cellular matrix (ECM) expression in relation to tissue repair and remodelling [10].

Gene expression profiling successfully identifies many genes with known function. Of the 30,000 + genes that have been identified, only a handful have a defined function. Therefore the next task is to define a function for each new gene and to determine how the protein products interact at a cellular level and fit into the bigger picture of the cytokine networks.

This study is designed to examine gene expression within the kidney allograft at the time of transplantation, in an attempt to predict which patients will develop rejection or graft dysfunction in the long-term. This data will also provide information on biomarkers that can be associated to other end points such as chronic rejection and chronic allograft nephropathy. This will enable us to tailor anti-rejection drugs to every patient depending on their immune response, and identify potential non-invasive biomarkers for the monitoring of progression of these intra-graft events.

2 METHODS

2.1 Ethical Approval

Formal ethical approval was obtained from Nottingham Research Ethics Committee 2 (ref: C2040304), and from Research and Development at Nottingham City Hospital. This allowed pre and post transplant biopsies and blood samples to be taken from transplant recipients once informed consent had been obtained.

2.2 Tissue handling

Samples were obtained during bench preparation of the kidney prior to transplantation or during kidney biopsy following transplantation.

I. Surgical technique

The pre-implantation biopsy taken prior to either deceased or live donor kidney transplantation was taken using a size 15 scalpel blade. An elliptical wedge of tissue was taken with the approximate dimensions; 10mm in length, 2-3 mm wide and 4-5 mm deep. This sample shape and size was used in order to facilitate repair of the kidney and minimise bleeding from the biopsy site following reperfusion. Two vertical mattress sutures using a 3/0 vicryl suture were used to repair the defect.

The biopsy sample was placed into icy Soltran Kidney Perfusion Solution (Baxter Health Care, UK). At the earliest opportunity the sample was flash frozen using liquid nitrogen and then placed into a labelled sterile sample bag and placed into the -80°C freezer.

II. Needle biopsy procedure

Following consent biopsies were taken. Following the administration of local anaesthetic, using an aseptic technique a 16 Fr trucut biopsy needle was used to take the samples. Depending on the clinical scenario the pathologist required up to three cores of tissue (1-2 cores for histology and immunohistochemisry) and a further core for this study. It was important that the biopsy specimen to

be used for research was **not** placed into formaldehyde for macroscopic assessment, as this would diminish the integrity of RNA [139]. The sample was placed into a 1.5ml PCR tube containing 500µl RNA*later*® *and* transferred to the -80°C freezer.

III. Tissue Storage.

There was variation in tissue storage techniques at different times during the research project. Prior to February 2004 the biopsied tissue was flash frozen in liquid nitrogen and transferred to the -80° freezer. Between February and April 2004 the sample was temporarily stored in icy Soltran, transferred to a sterile 2.5ml plastic vial containing 750 µl RNA*later*® and then placed in the -80° freezer. After April 2004; as above (Feb – Apr 2004) except that the tissue sample was transferred directly to a sterile 2.5ml plastic vial containing 750 µl RNA*later*® and then placed in the -80° freezer.

2.2.1 Deceased and Living Donor Biopsy Samples.

Deceased donor biopsy samples were taken following organ retrieval prior to transplantation. Living donor biopsy samples were taken following perfusion of the kidney with icy soltran immediately after laparoscopic donor nephrectomy.

I. Donor and Recipient Demographics.

	Tx date	Donor age	Donor type	Recipient age
E02	12-Nov-2003	43	LD	36.2
E03	14-Oct-2003	62	DD	65.6
E04	10-Dec-2003	58	LD	28.1
E05	24-Nov-2003	45	DD	39.1
E06	7-Jan-2004	28	LD	4.1
E07	3-Nov-2003	15	DD	21.5
E08	24-Apr-2002	50	LD	32.1
E09	23-Nov-2000	29	LD	2.3
E10	12-Dec-2003	44	LD	34.4
E13	7-Dec-2001	56	DD	47.6
E14	1-Jan-2002	20	DD	35.4
E15	11-Feb-2004	44	LD	14.6

E16	27-Mar-2002	49	LD	22.8
E17	9-Jan-2002	61	LD	64.4
E18	15-Jan-2002	21	DD	33.2
E19	15-Jan-2004	48	DD	50.9
E20	18-Jan-2002	17	DD	14.7
E21	5-Dec-2001	56	DD	51.8
E22	14-Sep-2001	37	DD	37.4
E23	5-Oct-2001	25	DD	60.7
E24	22-Jan-2004	27	DD	5.1
E25	6-Jun-2004	27	DD	37.2
E26	21-Apr-2004	53	LD	47.2
E27	26-Apr-2004	56	DD	62.5
E28	28-May-2004	20	DD	40.2
E29	21-May-2004	33	LD	4.7
E30	19-Apr-2004	35	DD	7.7
E31	19-Apr-2004	35	DD	42.0
E32	16-Jun-2004	59	LD	58.2
E33	18-Sep-1993	unknown	DD	49.9
E34	14-Dec-2003	44	DD	39.9
E35	14-May-2004	53	LD	23.2
E36	10-May-2004	53	DD	34.3
E37	17-Mar-2004	unknown	DD	34.2
E38	29-Jul-2004	unknown	DD	25.7
E39	17-Oct-2001	48	LD	20.6
E40	21-Nov-1999	unknown	DD	17.5
E41	10-Aug-2004	unknown	DD	35.1
E42	17-Aug-2004	unknown	DD	56.4
E43	17-Oct-2001	48	LD	20.6
E44	31-Aug-2004	unknown	DD	30.7
E45	14-Sep-2004	unknown	DD	52.8
E46	12-Feb-2004	37	DD	21.0

Table 3 - Donor and Recipient age and donor type.

DD = Deceased Donor, LD = Living Donor

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Table 3 shows the list of experiments, the type of donor, donor age and recipient age. 44 patients received kidney transplants, 28 were from deceased donors and 16 from living donors.

All			Adults			Children		
Donor	DD	37	Donor	DD	39	Donor	DD	26
	LD	47		LD	51		LD	34
Recipient	DD	38	Recipient	DD	41	Recipient	DD	9
	LD	28		LD	35		LD	6

Table 4 - Average Age in Years

The average age of donor was 37 for deceased donors and 47 for living donors (Table 4). The average age of recipients was 38 for deceased donors and 28 for living donors (adults) and 2.3 (children). The average age of donor was 43.6 (adults) and 30.4 (children).

2.2.2 Classification of Samples

For the purpose of this study tissue biopsies taken from patients were classified as shown in Table 5

Donor Source	Timing of Transplant Biopsy	
Deceased donor	Pre-implantation biopsy	Post - transplantation kidney biopsy taken for clinical reasons any time following transplantation
Living donor	Pre-implantation biopsy	Post - transplantation kidney biopsy taken for clinical reasons any time following transplantation

Table 5 - Table to show category of biopsy sample subdivided into donor type and the timing of biopsy

Donor Source

Transplant biopsy samples were sourced from deceased donors, where the kidney was retrieved from a person that had been diagnosed as being brain-stem dead, or from living donors where fit and healthy people donated one kidney to a relative or friend.

I. Timing (Pre-implantation)

The majority of samples were taken at the time of routine pre-implantation transplant biopsy. This biopsy procedure is taken routinely in order to provide the baseline histology of the graft on which to compare if subsequent biopsy are required for clinical reasons. The baseline histology also gives us a very generalised idea of the state of health of that kidney in order to give us some idea of what function we are likely to expect from the graft. For example a kidney from an 18 year old is more likely to perform better than a kidney from a 65 year old in which widespread arteriosclerosis or glomerular hyalinisation is seen.

II. Timing (Post-implantation)

Post-implantation biopsies were taken at any time following transplantation. They were taken for clinical reasons in order to diagnose the underlying cause of graft dysfunction. A research sample was taken when tissue excessive to the requirements for histological diagnosis was obtained.

III. Functional Categorization of Samples

A. Early Samples

Early transplant biopsy samples were taken within 4 weeks following transplantation. This group was sub-divided depending on the outcome (graft function).

i. Immediate graft function

A graft functioned immediately when recipient plasma creatinine started to fall on day 1 following transplantation and continued to do so until the recipient plasma creatinine fell to below 150 µmol/l.

ii. Moderate function

A graft functioned moderately when recipient plasma creatinine started to fall on day 1 following transplantation but failed to reach 150 µmol/l or below. If the recipient plasma creatinine started to drop after day one but then fell to 150 µmol/l or below these were also included in moderate function group.

iii. Poor function

A graft functioned poorly when recipient plasma did not fall on day 1 following transplantation and failed to reach 150 µmol/l or below.

iv. Non-function

In a non- functioning graft no fall in recipient plasma creatinine was seen at any time.

The pathophysiology causing graft dysfunction in the above groups was due to one of, or a combination of acute tubular necrosis (ATN), acute rejection, thrombosis/ischaemia, calcineurin induced nephrotoxicity or unknown cause.

B. Late transplant biopsy samples.

The purpose of distinguishing between early and late transplant biopsy samples was to identify pathological processes that were more likely at that time; early graft dysfunction was likely to be caused by ATN, acute rejection, thrombosis/ischaemia or calcineurin induced nephrotoxicity and not by chronic allograft nephropathy or chronic rejection and vice versa.

There were no late biopsies taken on transplanted grafts that originally performed with immediate function. This will be discussed later.

C. Protocol biopsies.

Some Transplant units perform routine “protocol biopsies”. These are done as part of that particular units management protocol or as part of a research project. When this is the case biopsies are taken at pre-determined times (e.g.

3, 6 and 12 months), regardless of the function of the graft. Protocol biopsies were not part of the management protocol or research protocol in the unit where this research took place. This fact in part explains why there were no late biopsies taken in the immediate function group; there was no clinical need and no routine (protocol) biopsies.

Of all 46 experiments 44 were hybridised with the same control RNA source. Because test samples were not being hybridised together this meant that less test sample would be lost should the hybridisation fail. Microarray data analysis software was designed to provide normalisation of samples based upon a common control sample. Therefore, as each test sample was hybridised with the same control sample comparison could be made across all test samples. The purpose of using a common control sample was to reduce inter and intra-experimental variation so that results obtained from any one hybridisation could be compared with any other sample using the same control.

IV. Control Sample

a) Pooled RNA control

During the development of the technique a pooled mRNA (Ambion) was used as a quality control. This was made into probes as described earlier along with sample RNA so that yields and quality control parameters could be compared to ensure that the extracted sample mRNA was being processed into probes suitable for hybridisation. These gene pooled probes were therefore not used for any subsequent hybridisation or analysis purpose.

b) Human RNA control

The control RNA used for probe preparation for the purposes of hybridisation was sourced from a human kidney that had been removed. It had contained a small renal tumour. The rest of the kidney was histologically normal and the patient had had normal renal function. The biopsy used to source the control sample was taken from the normal part of the kidney. This was deemed to be a far more suitable control because it was human and not manufactured. It was tissue taken from the same part (cortex) of the same organ; kidney.

The control sample had been immediately taken from the removed kidney and stored in RNA*later*® and therefore, experienced minimal physiological insult compared to the biopsies taken from deceased donor kidneys. With regard to the living donor kidneys the level of physiological insult was deemed to be more similar the main difference being the amount of warm ischaemic time the living donor kidney was exposed to (i.e. the amount of time the living donor kidney was without a blood supply prior to the biopsy sample being taken and stored in RNA*later*®).

Depending on the sample quality amplified RNA would provide enough material for between 1 and 8 hybridisations. Table 8 summarises experiments that were grouped dependent on the source of donor kidney; deceased or living and the clinical setting in which the biopsy sample had been taken.

LIVING DONOR	DECEASED DONOR	
E04/LD/IF E08/LD/IF E15/LD/IF E16/LD/IF E17/LD/IF E26/LD/IF E29/LD/IF E32/LD/IF	E05/DD/IF E07/DD/IF E13/DD/IF E14/DD/IF E21/DD/IF E22/DD/IF E23/DD/IF E24/DD/IF E27/DD/IF E30/DD/IF E36/DD/IF E44/DD/IF E45/DD/IF E46/DD/IF	Immediate Function
E35/LD/IF/AR	E38/DD/STF E19/DD/STF/#2 E47/DD/STF E37/DD/BX/EARLY	Moderate Function - Early
	E28/DD/ATN E31/DD/ATN E41/DD/ATN E42/DD/ATN	Poor Function - Early
	E25/DD/NF E34/DD/TXNEPH	Non Function - Early
	E33/DD/BX/LATE E40/DD/BX/ LATE	Moderate Function - Late
E39/LD/BX/ LATE E43/LD/BX(2)/OLD		Poor Function Late

Table 6 - Summary Table of Experiments.

Key:	LD – Living Donor	DD – Deceased Donor
	IF – Immediate function	NF - Non function
	ATN – Acute tubular necrosis	BX – Biopsy
	STF - Slow to function	TXNEPH – Transplant nephrectomy

In Table 6 the first column lists the experiments using the simple annotation E for experiment followed by a number 2 – 47. E1 was removed from this analysis; it was a separate experiment performed using Method B. It used two samples from the same patient at time of transplantation and then during acute rejection, therefore did not use the universal control. E1 was prepared up to the

stage where data analysis could be performed but for the purpose of this thesis it will be excluded. Instead the two test samples were hybridised with the control sample and this is reflected by experiments 46 and 47.

E11 and E12 were experiments performed during the development of the Method B and were performed with a different control RNA. This control (C1B) was the original control that was being used for the first 12 hybridisations. These however, produced poor results due to technical problems that will be discussed later. This control sample was used up in these initial hybridisations. At this point the control sample C2A was obtained, which had better characteristics in terms of RNA quantity and quality and it was decided that this should be the common control sample. E11 and E12 hybridised adequately, suitable for further analysis but will also be excluded in this thesis.

2.3 RNA extraction

All RNA extractions were performed using the RNeasy® Mini Kit. The kit was designed to isolate up to 100μg of RNA molecules longer than 200 nucleotides.

a) Quantification

Approximately 30 mgs of tissue was thawed. This was approximately a 3x3x3 mm^3 piece of tissue cut from the frozen wedge specimen that had been taken pre-transplant or at subsequent biopsy. It was important to note than any tissue not being used for RNA extraction was not allowed to defrost and was returned to the -80° C freezer immediately.

b) Lysis

The sample was placed in a 600 μl lysis buffer containing guanidine isothiocyanate (GITC). GITC served to denature the sample and inactivate RNases to maximise the isolation of intact RNA. It was important to add 10 μl of 14.3M β-mercaptoethanol per 1ml of lysis buffer at this stage as it was not included ready mixed in order to prolong the shelf life of the lysis buffer.

c) Disruption and Homogenisation

Sample disruption and homogenisation was performed using a Rotor-stator homogeniser. Complete disruption of cell walls and plasma membranes was required to release the maximum quantity of RNA. Homogenisation was necessary to reduce the viscosity of cell lysates produced by disruption. The disrupted/homogenised sample was visually inspected to make sure that there were no tissue fragments remaining. If there were the sample was further homogenised until no tissue was visible. Partial homogenisation would cause partial release of RNA. Therefore a partially homogenised sample would contain less RNA available to bind to the RNeasy binding membrane, significantly reducing RNA yield. The lysate was centrifuged at maximum speed for 3 minutes. The supernatant was added to 600 µl of 70% ethanol and mixed by pipetting. The alcohol was required to provide appropriate binding conditions.

d) Binding and Washing

This was then applied to an RNeasy mini column (filter cartridge and 2ml collection tube) in 700 µl aliquots and centrifuged at 10 000 rpm for 15 seconds. The flow-through was discarded following each spin. 700 µl of binding buffer was added to the RNeasy column and centrifuged for 15 seconds at 10 000 rpm. The filter column was replaced into a new 2 ml collection tube. The column was washed twice with 500 µl of wash buffer which was pipetted onto the RNeasy filter column. Each time it was centrifuged at 10 000 rpm for 15s. A further spin was performed to eliminate the chance of alcohol contained in the wash buffer being carried over.

e) Elution

To elute the RNA, the filter was placed into fresh 1.5 ml collection tube, 30µl RNase-free water was added and then the column was centrifuged. This was repeated with further 50µl RNase free water.

2.4 RNA Quality Control / Quantification techniques.

In the initial method prior to April 2004 the Pye Unicam UV2-100 Spectrophotometer was used in order to assess the quality (based on the absorbance curve and ratio), and the quantity (calculated from the absorbance and dilution factor.

Initial method - RNA quantity calculation.

5 µg of RNA was required in the next stage (amplification). This was calculated using the readings acquired from the spectrophotometer in the following formula:

40 x absorbance at 260 nm (A) x Dilution Factor = µg/ml

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample (eluted volume) = 50 µl

Dilution = 1µl of RNA sample + 99 µl distilled water (1/100 dilution).

Measured absorbance of diluted sample in a 100 µl cuvette; A = 0.048

Concentration of RNA sample = Extinction coefficient* x A x dilution factor

= 40 x 0.048 x 100

= 192 µg /ml

Total yield = concentration x eluted volume of sample (ml)

= 192 µg /ml x 0.05 ml

= 9.6 µg

*The Extinction coefficient for RNA diluted in water. An absorbance of 1 unit at 260 nm corresponds to 40µg RNA per ml.

5µg of sample RNA was required for cDNA synthesis and was calculated as follows:

Total yield = 9.6µg in 50µl

1µg = 50 µl / 9.6

5µg = 50 x 5µl / 9.6

= 26.0µl of sample

Initial method - RNA quality analysis.

Spectorophotometric measurement gave the 260/280nm absorbance spectrum. The 260/280nm ratio was used as mark of quality control where a ratio values between 1.8 and 2.1 indicated a sample purity of sufficient quality to continue with the probe preparation (Ambion).

New method - RNA quality analysis and quantification

It was imperative that accurate analysis of RNA quantity and quality was performed to assess the suitability of the extracted RNA for further processing.

a) Nanodrop Spectrophotometer

The NanoDrop Spectrophotometer [25](see figures 6 & 7) was designed to measure nucleic acid concentrations in sample volumes of one microlitre without the need for cuvettes. This is achieved by the surface tension that develops between the sample surface and the detector surface. Therefore a continuous column is formed which completes the optical circuit.

Figure 6 - Nanodrop sample application. With the sampling apparatus open, a droplet of sample is pipetted onto the lower measurement pedestal

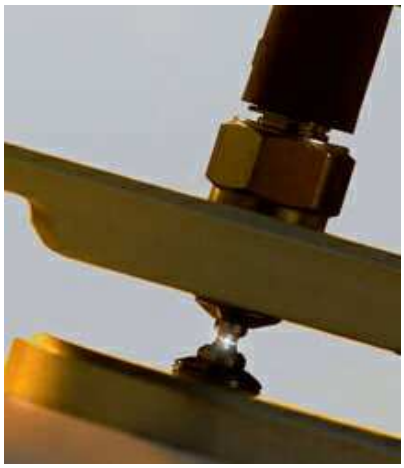


Figure 7 - Nanodrop droplet adhesion. When the sample apparatus is closed, the upper measurement pedestal slightly compresses the droplet. Surface tension holds the sample in place.[140]

The Nanodrop ND-1000 Spectrophotometer (Labtech, East Sussex, UK) required only 1µl for analysis which was very important as the extracted RNA volume was only 50µl. The developers recommended that a 1 or 2µl pipette is used to apply the samples as a poorly calibrated 10µl pipette could introduce volume error. Another benefit was that the test sample could be retrieved and used to determine the integrity of the RNA i.e. used on the Agilent Bioanalyser. This option was used at times when RNA quantities needed to be preserved. Another useful feature of the Nanodrop was the ability to measure Cye Dye incorporation into microarray probes prior to hybridisation. This will be discussed later.

b) Agilent 2100 Bioanalyser

The Agilent 2100 Bioanalyser (Figure 8) (Agilent Technologies, Cheshire, UK) has largely replaced gel electrophoresis in RNA sample quality control. It was used in order to standardise the assessment of RNA quality. Samples were prepared according to the manufacture's protocol: Agilent RNA 6000 Nano Assay Protocol [141] .

Figure 8 - Agilent Bioanalyser

The RNA Nano LabChip was used to analyse up to 12 samples at a time and is shown in Figure 9.

Figure 9 - Agilent Bioanalyser LabChip

2.5 RNA Amplification – Initial method

The following method is adapted from; Protocol RNA Amplification for use with Pan® Oligo Microarrays Version 220601E. The protocol incorporates the use of the following kits.

- cDNA Synthesis System (Roche)
- High Pure RNA Tissue Kit (Roche)
- MEGAscript T7 Kit

2.5.1 The Eberwine principle

The principle behind the method was based on the RNA amplification protocol developed by Dr. James Eberwine [135] . The technique utilizes an oligo(dT) primer containing the T7 RNA polymerase promoter for synthesis of first strand cDNA. The primer binds to the poly(A) tail at the end of mRNA (3' end). The technique is designed to only amplify the mRNA fraction of total RNA to produce double stranded DNA

2.5.2 cDNA Synthesis from total RNA using the cDNA Synthesis System (Roche)

I. First strand synthesis:

AMV reverse transcriptase was used for first strand cDNA synthesis. The initiation of the first strand synthesis depends upon hybridization of the primer Oligo [(dT) 24 T7promotor] 65 to the mRNA, usually at the poly (A) tail

A 5 µg of (sample) total RNA was pipetted into a 0.2 ml PCR tube. 2 µl (2 µg/µl). Oligo $[(dT)_{24}T7promotor]_{65}$ primer was added and then the total volume was made up to 21 µl. To denature the RNA the mixture was gently flicked to mix and placed in the thermocycler set at 70°C for 10 minutes. The tube was then placed on ice prior to prevent rebuilding before proceeding to the next step.

In a separate tube the following were mixed together:

- 8 µl of 5x RT buffer
- 4 µl 0.1M DTT
- 1µl RNase Inhibitor (25 U/µl)
- 4 µl dNTP mix (10 mM each nucleotide)
- 2 µl AMV reverse transcriptase (25 U/µl).

In practice a mastermix containing multiple amounts of each constituent was made up. For example if 10 samples were to be prepared 80 µl of 5x RT buffer would be used. In addition an extra 5% of each constituent was included (e.g. 84 µl 5x RT buffer) in the mastermix to allow for pipetting error.

To the sample tube (21 µl) 19 µl of the mastermix was added giving a total volume of 40 µl. The mixture was vortexed briefly and placed in a thermocycler at 42°C for 60 minutes. The tubes were again placed on ice prior to continuing on to the second strand synthesis stage.

II. Second strand synthesis

In a new tube the following reagents were combined:

- 5x 2nd strand-buffer 30 µl
- dNTP-mix (10 mM each nucleotide) 1.5 µl
- 2nd strand enzyme mix 6.5 µl
- redistilled. water 72 µl

Total volume =150 µl

A mastermix containing multiples of the above quantities was used. This was added to the first strand reaction (40 µl), vortexed and placed in the thermocycler at 16°C for 2 hours, then placed on ice.

Functions of the 2nd strand enzyme mix:

i. RNase H inserted nicks into the RNA, providing 3‘OH-primers for DNA polymerase I present in the enzyme mix.

ii. The 5‘→ 3‘exonuclease activity of DNA polymerase removed the primer stretches in the direction of synthesis, which were then replaced with new nucleotides by the polymerase activity. E.coli Ligase linked the gaps to complete the dsDNA.

Following the incubation 20 µl (20 U) T4 DNA polymerase was added and briefly vortexed.

iii. T4 DNA polymerase was added to remove any remaining overhanging 3‘ends on the dsDNA in order to blunt the terminal ends.

This was incubated at 16°C for 5 min and then the reaction stopped by adding 17 µl EDTA (0.2M pH 8.0).

III. Digestion of RNA

As the starting material was total RNA the protocol recommended that RNase was added to digest any residual RNA.

- Proteinase K was then added to remove inhibitors of transcription (RNase and restriction enzymes).
- µl (15 U) RNase I was added and incubated at 37°C for 30 minutes followed by 5.0 µl (3U) of Proteinase K and incubated at 37°C for a further 30 minutes.

2.5.3 Purification of dsDNA using the High Pure RNA Tissue Kit

A. Binding / Washing steps

At room temperature 10 µl β-mercaptoethanol was added to 1 ml binding buffer. As with preparation of the mastermix 10 µl β-mercaptoethanol was added to each 1 ml of binding buffer to cover all samples being prepared at that time e.g. 100µl β-mercaptoethanol was added to 10ml binding buffer to cover 10 samples.

i. The active ingredient in binding buffer one was guanidine-HCl which solubilised and denatured proteins

The dsDNA reaction mix was transferred to a new 2ml reaction tube and added to 800 µl binding buffer (containing mercaptoethanol) and 400µl absolute ethanol and mixed well by pipetting up and down until the solution became homogeneous.

700 µl of the reaction mix were pipetted onto a filter column and collecting tube which was then centrifuged at 8000rpm for 15 seconds, discarding the flow-though. The process was repeated with the remaining 700 µl.

B. Washing steps

The column was washed once with 500 µl of wash buffer 1, and then with twice with 500 µl and 300µl wash buffer 2. After each wash the column was centrifuged at 8000 rpm for 15 seconds and the flow-through discarded.

i. Wash Buffer 1 (contained 4,5 M guanidine-HCl, 100 mM sodium phosphate) removed transcription inhibitors.
ii. Wash Buffer 2 (contained 20 mM NaCl, 2 mM Tris-HCl and absolute ethanol) purified template DNA from remaining impurities

The final spin was performed at 14000 rpm for 1minute and then repeated to dry the column of excess alcohol.

C. Elution

The column was placed into a new reaction tube. 40 µl elution buffer (containing nuclease-free, sterile, double distilled water) was added to the centre of the filter and then centrifuged at 8000 rpm for 1 minute. This procedure was then repeated with a further 40 µl elution buffer. The eluate was combined and centrifuged at 14000rpm for 2 minutes.

The sample was immediately placed on ice. A 2 µl sample was taken at this point to be read on the spectrophotometer (or Nanodrop). Readings of sample concentration were taken which allowed calculation of the amount of sample required (100ng dsDNA) to proceed to transcription and labelling.

The formula when calculating dsDNA is similar to that described previously but here the extinction coefficient for dsDNA diluted in water is 50 i.e. an absorbance of 1 unit at 260 nm corresponds to 50µg dsDNA per ml.

For example,

Volume of dsDNA sample (eluted volume) = 80 µl

Dilution = 2µl of dsDNA sample + 8 µl distilled water (1/5 dilution).

Measured absorbance of diluted sample in a 10 µl cuvette; A = 0.100

Concentration of DNA sample = Extinction coefficient* x A x dilution factor

= 50 x 0.100 x 5

= 25 µg /ml ≡ 25 ng/ul

To calculate volume containing 100ng:

$$25 \text{ ng} = 1\text{ul}$$

$$1 \text{ ng} = \frac{1\ \mu\text{l}}{25}$$

$$1 \text{ ng} \times 100 = \frac{1\ \mu\text{l} \times 100}{25}$$

100ng dsDNA = 4µl

The volume containing 100ng dsDNA was calculated and pipetted into a 0.5 ml tube and dried in the speed vac at room temperature. It was important to note the orientation of the collecting tube during the drying stage in the speed vac. This was in order to visualise the dried pellet so that the reagents used in the next step could be added directly onto it minimising the risk of losing the pellet.

2.5.4 T7 Transcription and Labelling of dsDNA using the MEGAscript T7 Kit

The following reagents were added to 100ng of dried dsDNA:

• 10 x reaction buffer	2 µl
• ATP/CTP/GTP-mix stock solution (25mM each)	6 µl
• U-nucleotide stock solution (50mM)	2 µl
• Nuclease free water	3 µl
• Enzyme mix including T7 RNA polymerase	2 µl
• Cy3-UTP (5mM)(or Cy5-UTP)	5 µl
Total volume	20 µl

These reagents were added to the dried dsDNA and carefully mixed again by pipetting.

i. This stage involved reverse transcription of the dsDNA template using T7 RNA polymerase. At the same time cyanine 3-NHS-ester or cyanine 5-NHS-ester (Cy3-UTP or Cy5-UTP) was added so that the Cye dye was incorporated as transcription reaction occurred i.e. direct labelling.

The mixture was incubated in a water bath at 37°C for 16 hours.

2.5.5 Purification of labelled cRNA using High Pure RNA Tissue Kit

At room temperature the following reagents were added to the labelled cRNA:

- 80 µl RNase free water
- 400 µl binding buffer (containing mercaptoethanol)
- 200 µl absolute ethanol

The reagents were mixed and then transferred onto a spin column and centrifuged at 8000 rpm for 15 seconds, and the flow through discarded.

a) Washing steps:

This was performed using the protocol as described earlier following 2nd strand synthesis in order to remove unbound DNA, primers, enzymes, and salts from the labelled cRNA.

b) Elution:

This was performed in the same way as mentioned previously giving 80 µl of labelled cRNA. A 1µl sample was taken for spectrophotometer reading. The labelled cRNA was then stored in the -80°C freezer in the dark.

It was important to remember when handling Cye dyes or reagents containing them, to do it in low light levels in order to avoid degradation or bleaching of the dye.

The concentration of labelled cRNA was measured using the spectrophotometer and the volume of each sample containing 15 µg calculated in the same way as described previously.

2.5.6 Fragmentation of labelled cRNA

On ice 15 µg of labelled cRNA were combined for each sample and its control. With the early experiments this control was made in the same in exactly the same way as outlined above using human pooled gene RNA. The sample was made up to 48 µl with RNase free water (*some samples required concentrating in the speed vac). To this was added 12µl 5x Fragmentation buffer.

i. cRNA products of T7 transcription are long (between 2-5 kb) and were fragmented with fragmentation buffer.

The sample was mixed and briefly vortexed and then incubated in the thermocycler at 94°C for 15 minutes, and then placed back on ice.

2.5.7 Purification of labelled, fragmented cRNA:

100 µl RNase free water was added to the cRNA and mixed carefully by pipetting and then transferred onto a Microcon concentrator and centrifuged at 9000 rpm for 10 min, making sure that a small layer of liquid remained above the membrane filter, to avoid drying out which would potentially cause degradation of the cRNA. The filter was removed and placed upside down in a new 1.5 ml reaction tube and eluted by centrifugation at 2500 rpm for 3 min.

2.6 Hybridization

The eluate was dried in a speed vac (maximum temp. 30°C, kept dark). The pellet was re-dissolved in 10 µl hybridization buffer and incubated at 94°C for 3 minutes then placed on ice.

i. 10 µl was the re-suspension volume recommended to cover the printed area of the slide under a 22mm coverslip.

The mixture was carefully transferred to the microarray slide. The principle involved applying the sample to one corner of the coverslip in order to allow capillary action to suck the hybridisation mix to cover the appropriate area evenly and without bubbles. The slide was secured in a hybridization block and incubated in a waterbath for 42 hours at 42°C.

Washing

The slides were then washed in the following solutions preheated to 30°C for five minutes each:

- 2x SSC, 0.1% SDS
- 1x SSC
- 0.5x SSC

The decreasing concentration of SSC used in each solution provided washes with decreasing astringent properties. The slides were dried by placing them securely in 50 ml Falcon tubes and centrifuging them at 1600 rpm for 2 minutes (with a swing-out rotor).

The slides were then stored in the dark ready for scanning.

2.7 RNA Amplification – New Method.

aRNA amplification procedure using the Ambion Amino Allyl MessageAmp™ aRNA Kit (Amersham Biosciences, Bucks, UK) – Method B

i. This technique was based on the principle as described by Van Gelder et al [135] using the T7 Oligo (dT) Primer to synthesize cDNA by reverse transcription.
ii. Half quantities of reagents were used which had been found to give good aRNA yields

2.7.1 Incorporation of aaUTP

The Amino Allyl MessageAmp aRNA Kit incorporated the modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) into the aRNA during in vitro transcription. aaUTP contains a reactive primary amino group on the C5 position of uracil that can be chemically coupled to N-hydroxysuccinimidyl ester-derivitized reactive dyes (NHS ester dyes) in a simple, efficient reaction (see Figure 10). Once purified, the dye labelled aRNA can then be used for microarray hybridization.

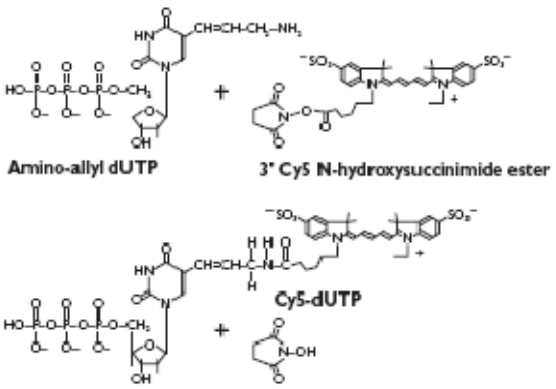


Figure 10 - Amino Allyl Labelling Reaction

Taken from Amino Allyl MessageAmp™ aRNA Kit manual (version 0503)

Figure 11 is a diagrammatic overview of the amino allyl message amp a RNA procedure.

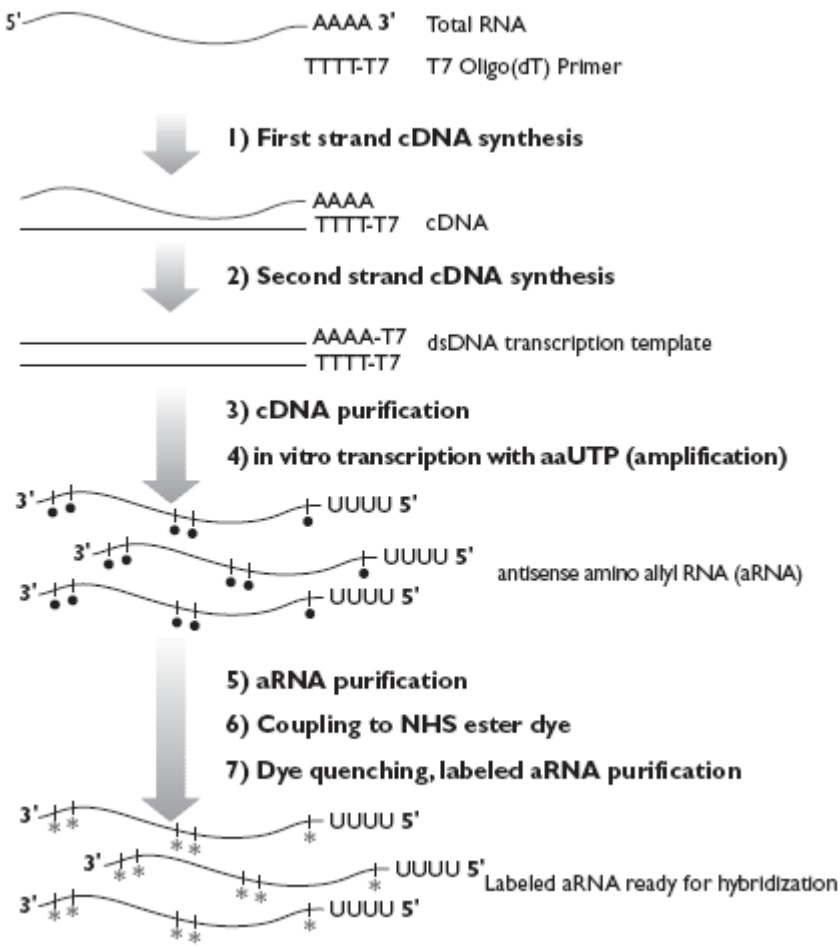


Figure 11 - Amino Allyl Message Amp Procedure [142]

2.7.2 First strand cDNA synthesis

1 µg of total RNA was placed into a sterile RNase-free microfuge tube. 0.5 µl of T7 Oligo(dT) Primer was added and the final volume made up to 12 µl with nuclease-free water and incubated for 10 min at 70°C in a thermal cycler.

i. The (total) RNA was primed with the T7 Oligo(dT) Primer to synthesize cDNA with a T7 promoter sequence (at the 5' end) by reverse transcription.

The RNA samples were centrifuged briefly (~5 sec) to collect the sample at bottom of tube and then placed on ice.

Sufficient reverse transcription master mix was prepared to synthesize first strand cDNA from all of the RNA samples in the experiment. An extra 5% of all reagents was calculated to cover pipetting error. The following reagents were added (single reaction):

- 1 µl 10X First Strand Buffer
- 0.5 µl Ribonuclease Inhibitor
- 2 µl dNTP Mix
- 0.5 µl Reverse Transcriptase
- 0.5 µl Spike mix

The mixture was mixed by gently pipetting up and down and centrifuged briefly to collect the master mix at the bottom of tube and placed on ice.

Lucidea Universal ScoreCard

Lucidea™ Universal ScoreCard™ was used to validate and normalize experimental data and to compare microarray data across experiments. It contains a set of 23 artificial genes designed from yeast intergenic regions which function as universal references that are consistent between experiments.

Contained within the scorecard are different categories of control:

i. Calibration controls allow evaluation of the dynamic range and sensitivity of the system.
ii. Ratio controls are used as references when measuring expression levels (up to 2.5 orders of magnitude.
iii. Utility controls allow the efficiency of the mRNA purification process to be assessed.
iv. Negative controls allow evaluation of signals produced by non-specific hybridization.

4 µl of reverse transcription master mix was added to each RNA sample mixed thoroughly and placed in the incubator set at 42°C for 2 hrs. After two hours the tubes were centrifuged briefly and placed on ice.

2.7.3 Second strand cDNA synthesis

Single stranded cDNA was converted using the T7 promoter primer into full length double stranded DNA (dsDNA), the transcription template.

On ice, the following second strand cDNA synthesis reagents were added to the reaction mix (quantities are given for a single reaction):

- 10 µl cDNA sample
- 31.5 µl Nuclease-free Water
- 5 µl 10X Second Strand Buffer
- 2 µl dNTP Mix
- 1 µl DNA Polymerase
- 0.5 µl RNase H

The mixture was mixed by gently pipetting up and down and centrifuged briefly to collect the master mix at the bottom of tube and placed on ice. Then the reaction tubes were incubated at 16°C for 2 hours.

2.7.4 Purification of dsDNA

i. The cDNA purification procedure removed enzymes, salts, unincorporated dNTPs and RNA from the cDNA sample. This eliminated the enzymatic digestion step that was used in the old method, to degrade RNA.

Before beginning the cDNA purification, the nuclease-free water was preheated to 50°C. One cDNA filter cartridge was firmly seated in a 2 ml wash tube and 50 µl cDNA Binding Buffer pipetted onto the filter and incubated at room temperature for 5 min.

ii. If a precipitate was visible in the cDNA Binding Buffer it was re-dissolved by warming the solution to 37°C and then allowed to cool to room temp before use.

A further 250 µl of cDNA binding buffer was added to each cDNA sample and mixed thoroughly. This was pipetted onto the centre of a cDNA filter cartridge

and centrifuged for 1 min at 10,000g. The flow-through was discarded and the cDNA Filter Cartridge replaced in the 2 ml wash tube.

500 µl of cDNA wash buffer was added to each cDNA filter cartridge and centrifuged for 1 minute at 10,000g and the flow-through discarded. The cDNA filter cartridge was spun for an additional minute to remove trace amounts of ethanol then transferred to a cDNA elution tube. To the centre of the filter 9 µl of nuclease free water (preheated to 50°C) was applied. This was incubated at room temperature for 2 min and then centrifuged for1.5 min at 10,000g. The procedure was repeated with a second 9 µl of nuclease-free water giving ~14 µl of double-stranded cDNA.

2.7.5 In vitro transcription to synthesize aRNA

i. This was the amplification step. The amino aaUTP generated multiple copies of amino allyl modified aRNA from the double-stranded cDNA template.

According to the protocol the inclusion of aaUTP by in vitro transcription had only minor effects on the reaction efficiency and yield. Additionally, since the incorporation of aaUTP by in vitro transcription was virtually identical in different samples, and since the dye coupling reaction was efficient and reproducible, labelled samples should not have the biases that could result from direct incorporation of modified nucleotides.

The following transcription reaction components were assembled (as a master mix) at room temperature, mixed and briefly centrifuged.

- 7 µl double-stranded cDNA
- 1.5 µ aaUTP Solution (50 mM)
- 6 µl 12 µl ATP, CTP, GTP Mix (25 mM)
- 1.5 µl 6 µl UTP Solution (50 mM)
- 2 µl 4 µl T7 10X Reaction Buffer
- 2 µl 4 µl T7 Enzyme Mix

The transcription reactions were incubated for 14 hrs at 37°C in an air incubator. An optional DNase treatment was used to remove template cDNA from the aRNA. 2µl DNase I was added to each reaction, gently mixed, then centrifuged briefly o collect the reaction at the bottom of tube and incubated for 30 min at 37°C.

2.7.6 aRNA Purification

78 µl of nuclease-free water (pre-heated to 50°C) was added to each aRNA sample to bring the final volume to 100 µl. This was mixed thoroughly. 350 µl of aRNA binding buffer was added to each aRNA sample, and mixed thoroughly. 250 µl of ACS grade 100% ethanol was added to each aRNA sample and mixed thoroughly.

The mixture was pipetted onto the centre of the filter in an aRNA filter cartridge and centrifuged for 1 min at 10,000g. 650 µl aRNA wash buffer was added to each aRNA Filter Cartridge and centrifuged for 1 min at 10,000g. The flow-through was discarded and the aRNA Filter Cartridge replaced in the aRNA collection tube and spun for an additional ~1 min to remove trace amounts of ethanol. The filter cartridges were transferred to a fresh aRNA collection tube and 50 µl of pre-heated nuclease-free water pipetted onto the centre of the filter. After 2 min the tubes were centrifuged for 1.5 min at 10,000g, and the elution repeated with a further 50 µl of nuclease-free water.

The concentration of the purified aRNA was determined using the Nanodrop Spectrophotometer before continuing to the next step: the dye coupling reaction.

2.7.7 Amino Allyl aRNA: Dye coupling and clean-up.

5 µg aRNA was taken and concentrated to a volume of 7µl by one of two methods:

i. Sodium acetate precipitation
ii. Vacuum dried

I. Precipitation method

The appropriate volume containing 5 µg aRNA (A) was added to one-tenth of this volume of 3M sodium (1/10A=B). To this, two times the sum of the volumes of aRNA and Na acetate was added 100% ethanol i.e.

$$\text{Volume 100\% ethanol} = 2x\ (A+B)$$

This was mixed and placed on ice for 20 minutes to allow the precipitation of aRNA to occur. The tube was spun for 15 minutes to collect the precipitant as a pellet. It was important to note the orientation of the tube/pellet. The supernatant was removed taking care not to remove the pellet. The pellet was washed with 200µl of 70% ethanol and the supernatant discarded. A further 200µl of 70% ethanol was added and the tube spun for 5 minutes at 10,000g and 4°C and then the supernatant removed. The pellet was vacuum dried until any residual alcohol had evaporated. The volume of each aRNA sample was made up to 7 µls with nuclease free water. 9 µl of coupling buffer was added and mixed well.

2.7.8 NHS Ester Dye Preparation:

4µl of DMSO was added to each vial of Cye Dye (Amersham Biosciences). The ester dye was added to the aRNA coupling buffer mixture, mixed well and incubated for 30 min at room temp in the dark. 4.5 µl 4M Hydroxylamine was added, mixed and incubated for 15 min at room temp in the dark. This was to quench the amine-reactive groups on the un-reacted dye molecules.

2.7.9 Dye Labelled aRNA Purification

The aRNA purification procedure described previously was repeated. The filter in the aRNA Filter Cartridge generally acquired the colour of the fluorescent dye during the purification. This was from the labelled aRNA binding to the filter. Most of the colour disappeared when the purified aRNA was eluted.

The sample was stored at –20°C in the dark. The dye labelled aRNA will be referred to as the Probe.

2.7.10 Probe Preparation

For each slide to be hybridized, a sample of each labelled probe (one cy3, one cy5) containing 40pmol of dye labelled cRNA was placed together in a 0.5 ml tube. Nuclease free water was added to give a total volume of 48 µl. 2 µl of Cot I DNA (to reduce non specific hybridization) and 50 µl of MWG 2x Hybridization Buffer was added bringing the total volume to 100 µl. The sample was kept on ice until required.

2.8 The Microarray

A. Initial Method

Custom designed microarray slides were used in the original experimental design. These had been developed in conjunction with MWG biotech. They contained 116 oligonucleotide spots. For reasons discussed later in section 0 (Rationale for changes in technique), the use of the slides was abandoned and no analysis was performed on the data acquired from these slides. For these reasons the slide design and choice of oligonucleotides will not be described or discussed.

B. New Method

In conjunction with the Department of Immunology at Nottingham university "in house" microarray slides were used. In contrast to the customised slide used in the initial Method, the in house slide was manufactured on site and contained 32488 oligonucleotides to represent the human genome and more hypothetical genes. It was subject to vigorous quality control [143].

I. Microarray printing and rehydration

Arrays were printed using a Biorobotics Microgrid II 600. The Microgrid II takes 108 slides in four trays, and can hold 24 x 384 well plates within its biobank. The printing pins used were Matrix 2500 pins, which give ~80 micron diameter spots. The human array was printed with 48 pins in a 4x12 array. The slides used were Schott Nexterion A+ (gamma APS coated) with a laser etched barcode [143].

Once the oligonucleotide spots were printed onto the glass slide the following protocol was used to rehydrate the spots so that they appeared as spots or discs with an equal density of oligonucleotide throughout each spot. Sub-optimal rehydration would lead to the spots appearing doughnut shaped.

Using powder free gloves the slides were placed array side down into the re-hydration tray and place into the humidifier (set at approx. 70% humidity) for 5-10 min, until full hydration was achieved. The slides were snap dried by briefly placing them onto a heating block (array side up) for 2-3 seconds and placed back into the plastic slide holders. Then they were placed into an oven for 3 hours at 70-80°C. Whilst the slides were heating, dishes for washing the slides was prepared. The first was left empty; the next two filled with ultra pure water and the final one with 95% ethanol.

To 335ml of 1-methyl-2-pyrrolidinone 5.5g of succinic anhydride was added and dissolved. Once dissolved 15ml of 1M sodium borate (pH8) was added. This buffered blocking solution was poured into the first glass slide dish and the slides plunged rapidly into it vigorously shaken, keeping the tops of the slides under the solution for approximately 1 minute.

The slides were placed onto a shaker in their dish and shook gently for 15 min. Excess blocking solution was drained off then the slide rack transferred into the first dish of water and gently agitated under the water for a few seconds. The rack was quickly transferred into the second dish of water and then the ethanol and the procedure repeated. The dish was centrifuged for 1 minute at 550 rpm. The slides were checked to be clean and dry then stored in plastic slide holders.

II. Hybridization

Hybridization was performed using the Tecan TX100 hybridization station (see figure 12). It was capable of hybridizing 12 microarray slides at a time. The standard manufacturers' protocol was used. This was a semi-automated machine that required the use of a computer and some user input.

Figure 12 - Tecan HS4800 Automated hybridisation station

The hybridisation was done in 9 steps taking about 4 hours. Step 1 involved the initial wash performed at 50°C in the most astringent SSC buffer. In step 2 the probe was injected into each sample well. Step 3 involved hybridization at 50°C. Steps 4-8 were further washes in buffers with decreasing concentration. Finally the slides were dried at 23°C (step 9).

III. Image Acquisition - Agilent Scanner

The Agilent microarray scanner (see figure 13) is a laser-induced fluorescence scanner designed to read microarrays deposited on standard 1 in x 3 in slides. The scanner measures the fluorescence intensity of labelled sample nucleic acid (DNA and RNA) bound to probe arrays. It is able to measure fluorescence from two dyes simultaneously required for differential gene expression studies. This technology provides for rapid, high-quality, automated, “hands-off” scanning of microarrays.

Figure 13 - Agilent Automated scanner

Up to 48 slides can be mounted in the scanner and then left to scan each slide automatically, typically taking 3-4 minutes per slide. The scanner needed to be connected to a computer with a Pentium® III 800 MHZ processor and 512 MB RAM, running Windows® 2000 with SP2 and Internet Explorer 5.5 or later were minimum requirements.

a) Lasers

The microarray scanner used a SHG-YAG laser (532nm) and a helium-neon laser (633 nm). The lasers excited Cyanine-3 (Cy-3) and Cyanine-5 (Cy-5) labelled RNA. The scanner was optimized for high signal-to noise performance in the Cy-3 (550—610 nm) and Cy-5 (650—750 nm) emission channels, with a wide dynamic range (four orders of magnitude) and low spectral cross-talk. This allowed for measurement of a very broad range of gene expression levels and for higher data confidence at lower signal levels.

b) Scanning

The laser excitation was scanned rapidly back and forth across the microarray. Movement of the microarray in the orthogonal coordinate was accomplished by

the slow linear stage. The dynamic/tracking auto-focus assembly kept the slide in focus with the scan lens ensuring the microarray was always positioned in the detection plane. These features maximise the signal-to-noise ratio by providing superior linearity, uniformity, and noise performance while scanning across the microarray surface.

c) Fluorescence detection

Fluorescent emissions from the labelled samples are detected using a high-performance PMT design. Very low noise amplifiers and analogue-to-digital converters process the PMT signal with a high signal-to-noise ratio.

IV. Feature Extraction and Analysis - Genepix Pro

The scanned image is saved as a large “raw image” TIFF (Tagged Image File Format), or rather a multi image TIFF, which contains two separate images scanned at 532 and 635 nanometers. One image file (containing two images) is obtained per experiment. This is opened into genepix version 6.1. The Gal file is then loaded onto the image see Figure 14. The GAL file contained data relating to each spot on the array.

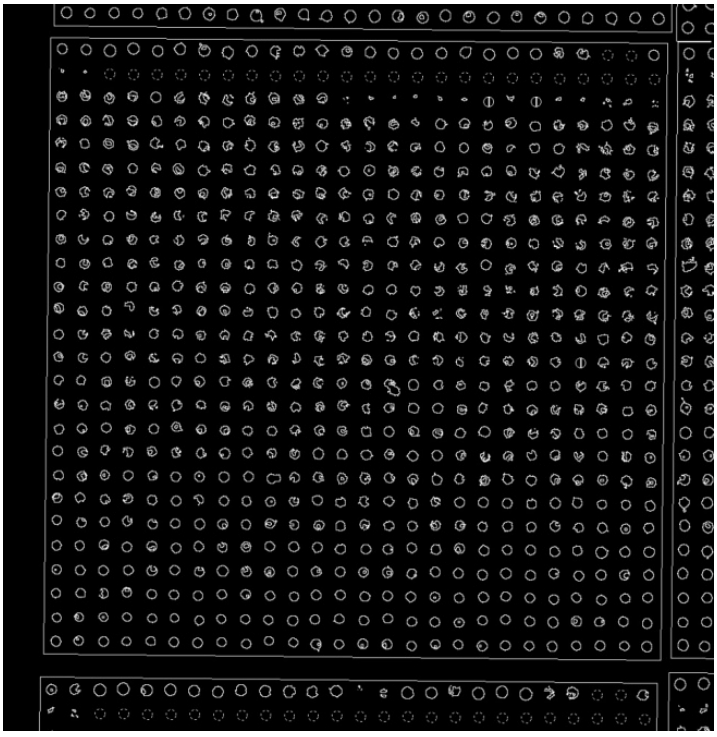


Figure 14 - Image of Gal file following alignment to the microarray. Only one block is shown for detail

Each of the 48 grids (containing 26 x 26 spots) needs to be accurately lined up to the overlying grid containing the Gal file information. Once the grid is aligned the option to "find irregular features" is checked. This allows the software to pick up spots on the array which are not circular and would otherwise be ignored; causing loss of potentially significant data. The decision to find irregular feature is based on the fact that when an array slide is scanned prior to hybridisation the spots are not all exactly circular. Sometimes the spot appears irregular or doughnut shaped. This variation is due to the slight variability in the conditions in which the array slides are processed and hydrated (see section 2.34). This variability in appearance of spots is not thought to affect the hybridisation process and hence the signal picked up by the scanner and subsequently by genepix pro, as long as the "find irregular features" box is checked.

The next stage involves genepix extracting information on each of the spots on the array including:

- spot size and circularity
- spot intensity
- background intensity

} mean and median values.

- etc, etc

The resulting file known as genepix results file (GPR file) was stored. This file contained the raw data that was compatible with microarray analysis software packages such as J-Express Pro and Pathway Assist.

2.9 Analysis Methods.

A. Significance Analysis of Microarrays

Significance analysis of microarrays (SAM) is a statistical testing algorithm developed specifically for microarray data analysis. It identifies induced and repressed genes with significantly different expression across samples [144]. It compares 2 or more samples to the control sample where the control sample is presumed to have a relative expression of 0. SAM takes into account the large number of genes in microarray dataset and assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements [144]. During SAM the data for each gene are permutated and a test statistic delta value is computed for both the original and permuted data for each gene[144] . SAM was used to identify significant genes based on differential expression between groups of samples created depending on clinical scenario and the control sample. For example, one could look at differential gene expression between deceased and living donor kidneys that have immediate function compared to the control. SAM is useful in that it gives an estimate of the False Discovery Rate (FDR) which is the proportion of genes likely to have been identified by chance as being significantly differentially expressed. SAM has an interactive interface which allows the distribution of the test statistic to be altered and allows the threshold for statistical significance to be set (through the tuning parameter delta) so that for example we allow a FDR of 0.05 – the genes identified have a 5% chance of being discovered by chance. If very large numbers of genes are identified at this level the FDR can be reduced e.g. to 0.01, i.e. the genes identified have a 1% chance of being discovered by chance. SAM generates a plot of the observed versus expected delta values where delta is the vertical distance (in graph units) from the solid line of the slope (which represents observed = expected).

B. Hierarchical Clustering

Hierarchical clustering was described by Johnson in 1967 [145]. Each sample (gene) is assigning to its own cluster. The closest (most similar) pair of clusters

are merged into a single cluster, so that there is one less cluster. Distances (similarities) are computed between the new cluster and each of the old clusters. This process is repeated until all items are clustered into a single cluster of size N. The last step can be done in different ways. In single-link clustering the shortest distance between a member of one cluster and another cluster is computed. In complete-link clustering the longest distance between one cluster and another cluster is measured. In average-link clustering, the average distance between each member of one cluster and each members of another is calculated. Average link clustering was used in this analysis.

C. Gene Ontology.

Gene ontology is a collaborative research tool developed to construct and use ontologies to facilitate the biologically meaningful annotation of genes and their products[146, 147] . Traditional methods e.g. hierarchical clustering, analyse data on a gene by gene basis and require time-consuming literature searching. GO annotations have already assigned biological function to genes. Genes are subsequently grouped by process e.g. Apoptosis, mitosis glucose transport etc. Therefore, genes involved in the same process with similar / different expression patterns are examined. GO terms are organised and related in a hierarchical manner.

GO captures information about the:

i. function of gene products
ii. where and when it acts
iii. its regulators and what it regulates

GO terms are divided into three parts:

A. Cellular component (CC)
B. Molecular function (MF)
C. Biological process (BP)

Cellular Component, describes locations, at the levels of sub cellular structures and macromolecular complexes. Examples of cellular components include ‘cell membrane and nucleus, with several subtypes of these complexes represented.

Molecular Function, describes activities, such as catalytic or binding activities, at the molecular level. GO molecular function terms represent activities rather than the entities that perform the actions and do not specify in what context the action takes place. Examples of individual molecular function terms are the broad concept ‘catalytic activity’ and the more specific ‘hydrolase activity’, which represents a subtype of catalytic activity.

Biological Process, describes biological goals accomplished by one or more ordered molecular functions. High-level processes such as ‘cell death’ have both subtypes, such as ‘apoptosis’, and sub processes, such as ‘release of cytochrome c from mitochondria ’.

D. Pathway Studio

Pathway studio is an updated version of the software application. Pathway assist comes with a database of molecular networks assembled from scientific papers [148].

3 RESULTS

3.1 Rationale for change in initial experimental design

The original experiment design involved the use of customised MWG Biotech array slides which are described in section 2.34. The RNA was handled and the probes were processed using the protocol described in section 2.5 (RNA Amplification – Initial Method). RNA was extracted from the 28 tissue samples taken between March 2001 and May 2002 using the RNeasy® Mini Kit. The quantity and quality of RNA produced was measured using a Pye Unicam spectrophotometer. The analysis reading was given in graphical form, of the 260/280nm absorbance spectrum and the calculated 260/280nm ratio were used as quality control at the different steps of probe preparation. An example is shown in figure 15. Ideal ratios should be between 1.8 and 2.1. Less than 1.8 can indicate too much protein contamination and above 2.1 may imply degradation.

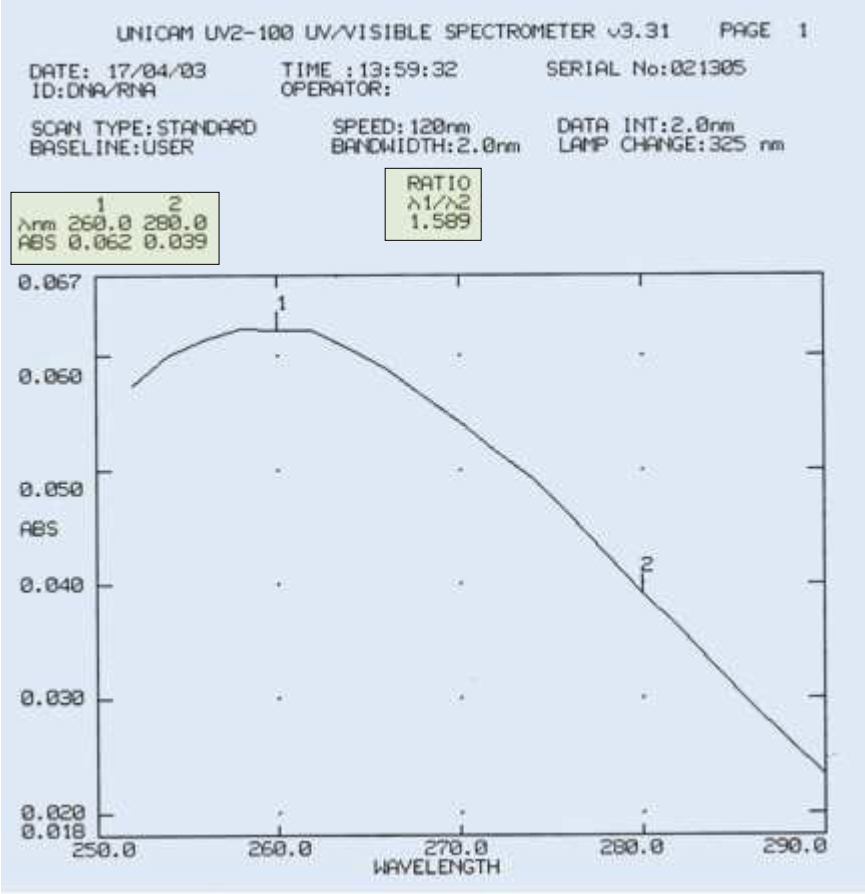


Figure 15 - reading from Sample 4 shows the typical spectrophotometric curve

It shows a good clean curve suggesting that the RNA is not significantly degraded or contaminated. However, the absorbance (y-axis) is very low suggesting that the quantity of RNA is very low in this sample. For this sample the absorbance was 0.062 and 0.039 at 260 and 280nm respectively. The resulting ratio was 1.589 which, unlike the curve suggests that this sample is of poor purity. The low reading and apparent poor quantity / quality of the samples might have arisen from poor calibration of the spectrophotometer. However, similar results were obtained before and after servicing and calibration of the equipment (see Table 7 & Table 8).

Sample No.	Elute (ul)	260	280	Ratio	Spec DF
1	50	0.017	0.011	1.513	70
2	50	0.034	0.023	1.469	70
3	50	0.057	0.034	1.67	70
4	50	0.085	0.052	1.644	70

Table 7 - Spectrophotometer readings taken following mRNA extraction using a 100µl cuvette prior to calibration

Sample No.	260	280	Ratio	Quantity in ul to=5ug	Conc (ug/ul)
1	0.009	0.006	1.589	138.9	36.0
2	0.020	0.013	1.563	62.5	80.0
3	0.048	0.030	1.569	26.0	192.0
4	0.062	0.039	1.589	138.9	248.0

Table 8 - Spectrophotometer readings taken following transcription mRNA→dsDNA with 100µl cuvette following calibration

This finding raised the possibility that the RNA quantity and quality was genuinely low in all of the samples. A second possibility which would account for the low readings was the need to use a x100 dilution factor with the cuvette. Whilst other users had found the machine reasonably reliable, they were using much larger quantities of sample material. This suggested that the degree of dilution was beyond the spectrophotometer accuracy limits. We next used a cuvette which required much smaller sample volumes allowing the use of a smaller dilution factor.

Sample No.	260	280	Ratio	Conc (ug/ul)
1	0.202	0.103	1.964	40.4
2	0.409	0.202	2.024	81.8
3	0.870	0.448	1.942	174.0
4	1.261	0.630	2.000	242.0

Table 9 - Spectrophotometer readings taken using the 10µl cuvette

The readings shown in Table 9 indicated that that use of this lower dilution factor gave different results. As expected the absorbance was higher. More importantly the A_{260} /A_{280} ratios were higher, between 1.96 and 2.02 which indicating that the purity of the RNA was good enough to continue with probe preparation.

This assessment of sample quality was applied to the full set of samples and again following transcription to dsDNA. Sample quality was assessed (see section – 2.12) and not processed further if the curve was not smooth or had any abnormal peaks. Examples are given in Table 10.

Sample No.	260	280	Ratio	Conc(ug/ul)	Yield(ug/ul)
1	0.097	0.080	1.202	24.3	1.94
2	0.062	0.048	1.294	15.5	1.24
3	0.100	0.082	1.218	25.0	2.00
4	0.094	0.071	1.333	23.5	1.88

Table 10 - Sample readings following transcription to dsDNA

The control and test sample readings of the dsDNA were similar to those shown in Table 10 i.e. having a ratio of 1.2 to 1.3. The low ratio suggested that there was a problem at some stage in the protocol. However, controls performed using the control RNA or DNA samples supplied with each kit gave similar results, and previous use of the above protocol by another researcher had given similar spectrophotometer readings and yields of DNA and RNA, and had been successful when used to make probes for microarray.

Therefore, test hybridisations were carried out using the samples that had been made using Cye labelled probes synthesised using the MEGAscript T7 Kit. Following hybridisation the array slides were transported appropriately to MWG Biotech for scanning. However, the slides showed no evidence of hybridisation.

The initial technique involved using a single channel microarray i.e. only one sample. Normalisation of the sample data was to be performed using statistical techniques. The starting concentration of dsDNA was not measured raising the possibility that much larger quantities of dsDNA were being hybridised leading to a "positive" result.

It may have been the case that the quantity or quality of sample being used was insufficient to hybridize successfully to the microarray slide, or hybridization may have occurred but too inadequate to be detected in the scanner. As part of the problem solving process it was thought prudent to test the integrity of the microarray slides.

Advice was sought from MWG Biotech. At their suggestion two samples with spectrophotometer readings to suggest that the starting RNA and final amplified dye labelled dsDNA (probe) was good in quantity and quality.

Sample No.	260	280	Ratio	Conc(ug/ul)	Yield(ug/ul)
14	1.11	0.58	1.90	2.23	22.3
16	1.65	0.92	1.80	1.51	33.1

Table 11 – Spectrophotometer readings of samples sent to MWG for quality testing and hybridisation

MWG performed quality control using the Agilent Bioanalyser and Nanodrop spectrophotometer. They found the probes to be good quality and so performed the hybridisation under their laboratory conditions. No evidence of hybridisation was found on the slides. Their next suggestion was to send some sample RNA for them to make a probe. This was also unsuccessful.

In order to exclude error arising from the use of reagents past their shelf life new kits were purchased and fresh reagents made.

Tissue that had been optimally extracted had now been used. New RNA extraction, transcription and amplification kits, had been used and MWG Biotech had quality control tested and repeated the experiments with no positive outcome. It seemed increasingly likely that the array slides may have deteriorated despite being stored as recommended by the manufacturer (in the dark at 5-8°C). MWG suggested testing more of the slides with their own probes which were "tried and tested".

In parallel to those developments, a review of the literature on RNA quality and tissue storage highlighted the use of RNA*later* as a well recognised and accepted storage media. All new tissue samples were stored in it from this point on

There were clearly fundamental problems with the experiment. Potentially the probe preparation process was not at fault and the array slide had deteriorated. However, during the fault finding process it became apparent that microarray technology had progressed. Microarray experiments were capable of representing the entire human genome and new bio analytical software was designed to work most efficiently with dual channel experiments.

It was recognised that single channel microarray experimentation would require additional biostatistical processing in order to analyse gene expression patterns. This would have involved normalisation of data being based only on sets of housekeeping genes built into the slide. The current trend in microarray analysis was to use dual channel microarray (test sample and control sample are labelled with different coloured dyes). Also, there had been much development in biostatistical software packages (e.g. Genepix Pro, J-Express) that were optimised for dual channel experiments using a control sample.

These facts led to the implementation of a new technique using two channels, (dye labelled test sample and a dye labelled control sample) hybridised onto a microarray slide containing oligonucleotide spots to represent the entire human genome.

In summary the change in methodological technique to method B was made for to a number of reasons. Logistic difficulties were encountered when seeking technical support form MWG Biotech. Hybridized slides had to be sent away for scanning, which was time consuming and highly expensive. The original customised array slide that had been developed by in conjunction with MWG Biotech contained a small number of oligonucleotides, not suitable for analysis; the aim of which was to identify changes in gene expression, and biological processes at a cellular level. Microarray technology had developed to a point where the entire human genome could be represented on one array slide. Method B was being developed at the Department of Immunology. Up to date, industry standard hardware was available for use and technical advice was at hand.

3.2 Qualitative and quantitative analysis - comparison of initial and new method.

All RNA extracts were tested on the Nanodrop spectrophotometer and Agilent Bioanalyser and a record of material suitable for further processing was made. 144 RNA extract were tested in this way resulting in only 46 deemed suitable for probe preparation.

The following are examples of readings of samples taken on the Nanodrop and Agilent machines to demonstrate good samples and samples which show varying degrees of denaturement. Figure 16 and Figure 16 demonstrate a good yield and good 18S and 28S peaks. Note the ratio in height between the peaks and the background and the control spike (of the RNA ladder) shown at 24 seconds.

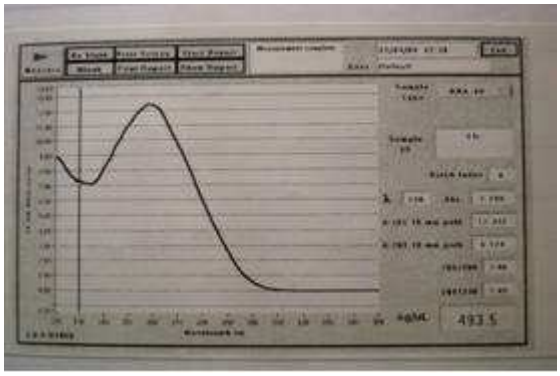


Figure 16 - Nanodrop reading demonstrating good yield

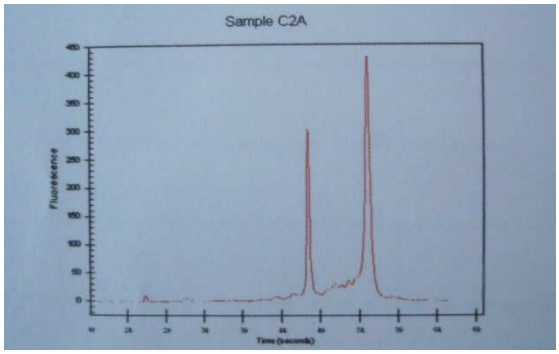


Figure 17 - Agilent reading demonstrating good 18S and 28S peaks

Nanodrop reading demonstrating good yield Agilent reading demonstrating good 18S and 28S peaks. The example shown in Figure 18 and Figure 19 is of a sample that is not as good in quality or concentration as the upper example but acceptable for probe preparation [149].

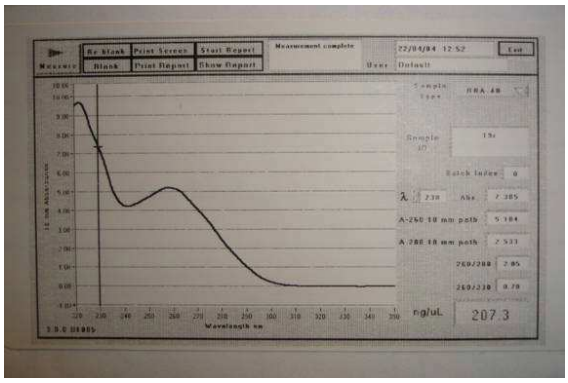


Figure 18 - Nanodrop reading demonstrating poorer yield

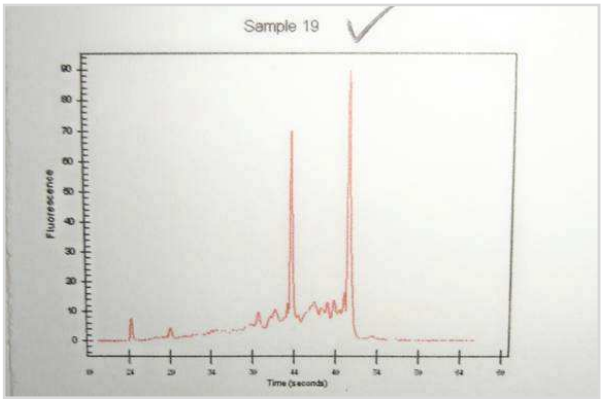


Figure 19 - Agilent reading demonstrating poorer but acceptable 18S and 28S peaks

In Figure 20 and Figure 21 it can be clearly seen in that this sample contained very little RNA. The trace from the Nanodrop shows a characteristic pattern suggestive of RNA degradation.

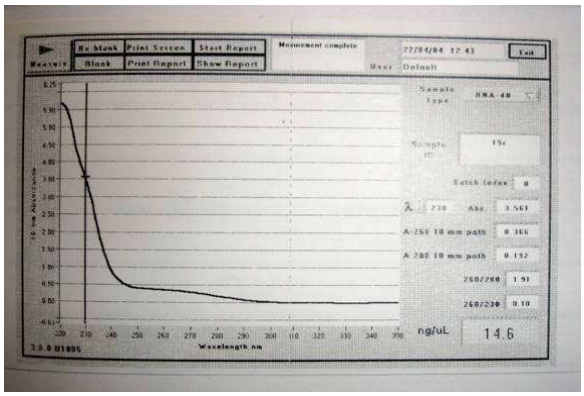


Figure 20 - Nanodrop reading demonstrating minimal yield

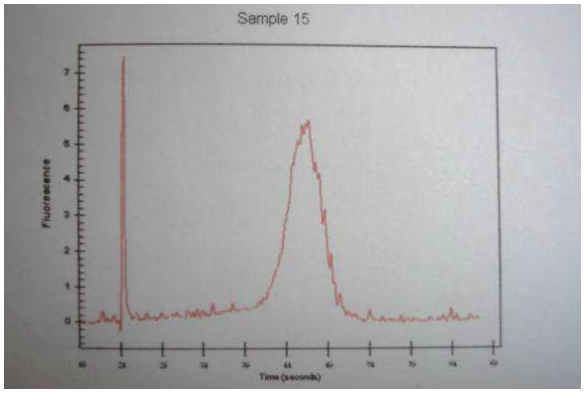


Figure 21 - Agilent reading demonstrating RNA degradation

In the example shown in Figure 22 and Figure 23 there is very little if any RNA and it only contains degraded material.

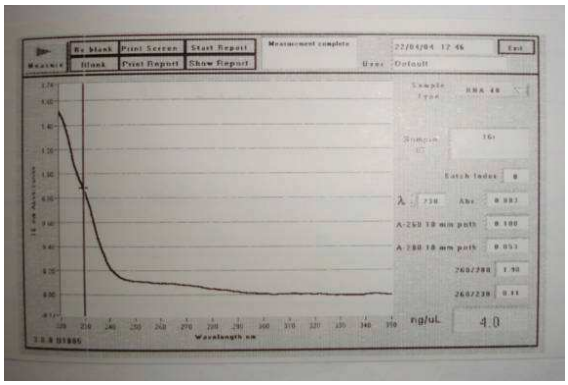


Figure 22 - Nanodrop reading demonstrating no yield

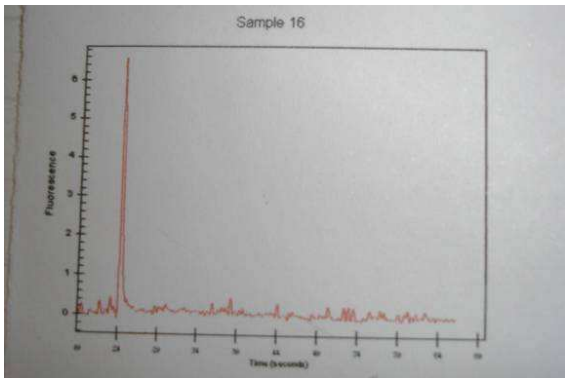


Figure 23 - Agilent reading demonstrating loss of RNA

The minigel shown in figure 23 is an example of RNA that had been extracted from tissue that had not been stored in RNA later. Samples 43 and 44 have identifiable 18S and 28S bands, however, there are also a lot of background bands that suggest degradation. It is an example of electrophoresis performed on samples that had been stored without the use of RNA later.

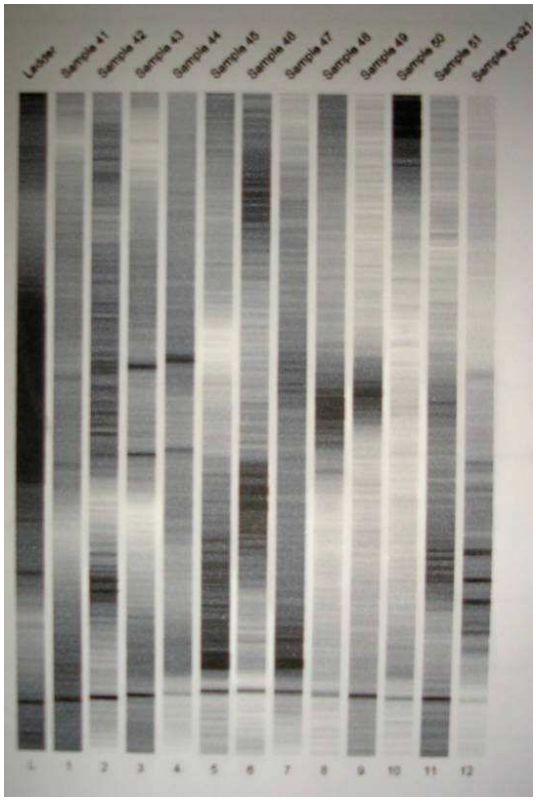


Figure 24 - Minigel demonstrating degraded RNA extracted from tissue not stored in RNA later

In contrast all of the samples apart from samples 15 and 16 show clean well demarcated 18S and 28S bands indicating preserved RNA. All of these samples had been stored using RNA*later*®. The trace from samples 15 shows a poorly demarcated, wide central band indicating poor sample quality. Sample 15 was taken from a deceased sourced kidney that did not function; its blood supply had thrombosed, therefore, this sample would represent necrotic tissue. Sample 16 shows signal across the entire range indicating RNA degradation. This sample was taken from a living donor kidney and not stored in RNAlater®; its electrophoretic trace suggesting that significant RNA degradation had taken place.

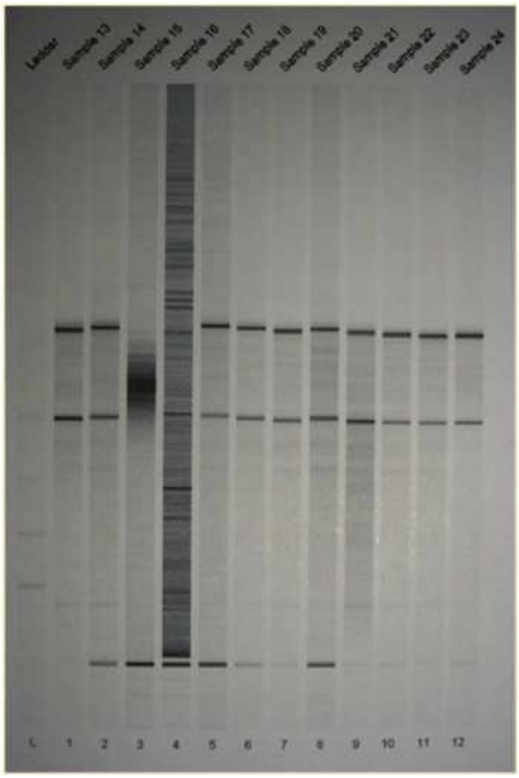


Figure 25 - Agilent minigel demonstrating samples containing mainly preserved RNA.

In figure 25 it can be seen from the minigel that the control samples contained good 18 and 28S bands suggesting that they contained intact RNA. Sample C2A was used for all hybridisations used in the analyses included in this thesis.

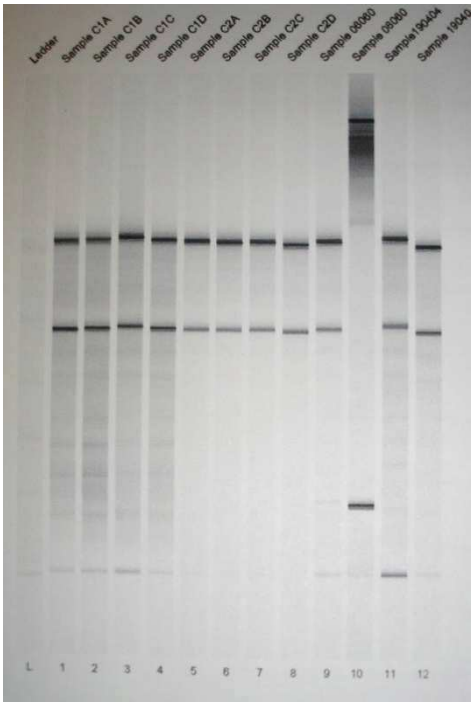


Figure 26 - Agilent minigel demonstrating RNA from control samples

3.3 Data quality control: visualisation, processing, filtering and normalisation

Genepix 6.1

Genepix 6.1 was used. A new experiment was created by opening the Spotpix suite. The Genepix results files (gpr files) were added. The corresponding jpeg image file was then added to each experiment. The data channels were automatically detected (F635 Median – B635 and F532 Median – B532). The option to combine in array replicates was chosen. This meant that oligonucleotides were represented on the slide more than once then the median value of the expression data was calculated. Resultant data was outputted on a logarithmic scale (log10).

Quality Control

I. Background

Figures 26 and 27 are taken from J-Express. It shows the ($\log_{10}$) intensity of background signal from the red channel (B635) and the green channel (B532).

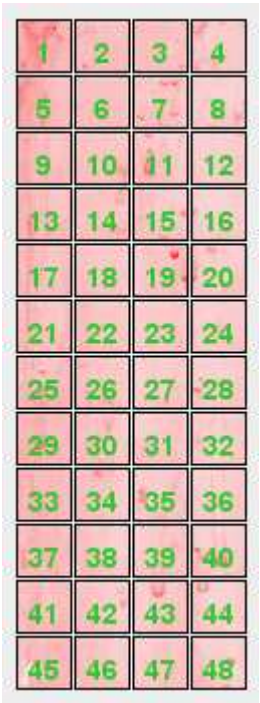


Figure 27 - (log10) intensity of background signal from the red channel (B635)

Figure 28 - (log10) intensity of background signal from the green channel (B532)

Figure 27 reveals the distribution to be fairly uniform apart from areas seen in blocks 1 and 5. Smears for example, shown in block 19 were used to identify areas of the array which needed closer scrutiny. These areas were inspected at high magnification and if the smear was judged to be artefact the spots were highlighted to be excluded from analysis.

II. Flagged spots, landing lights and the scorecard.

The quality control view was also used to visualise the distribution of spots flagged up as “not found” by genepix. Removal of flagged spots was done to improve the normalization and ratio estimates in microarrays. The flagged spots are shown in red in figure 28.

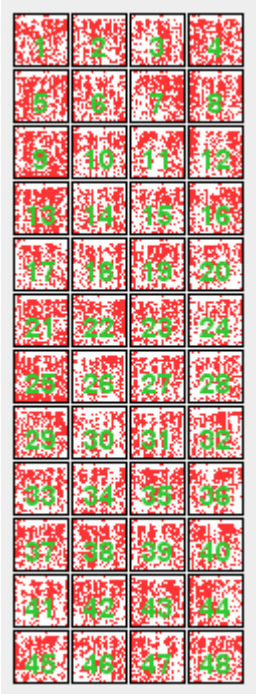


Figure 29 - Flagged spots that are not found by J-Express are shown in red.

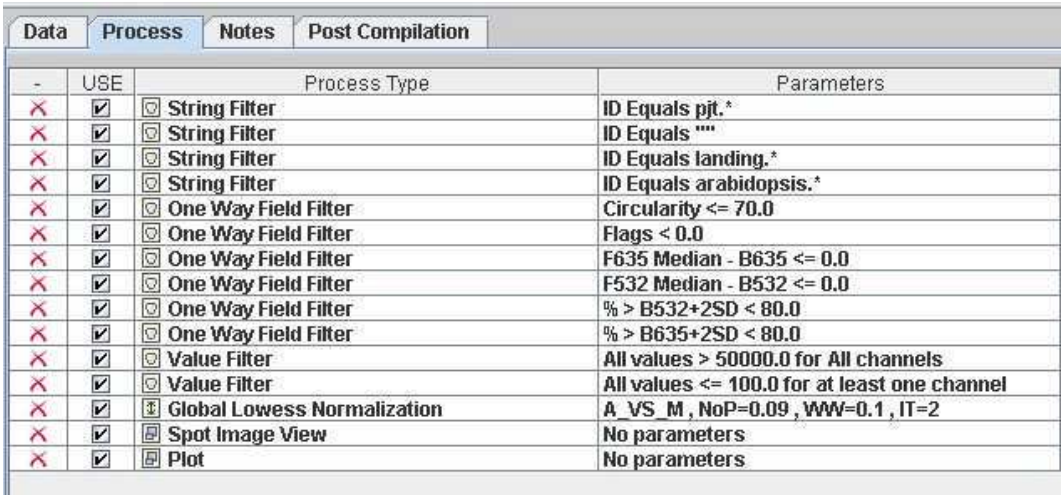
Figure 29 shows that the flagged spots were reasonably evenly distributed indicating that the microarray hybridisation had been uniform i.e. there were no particular areas of the slide that had not hybridised. Interestingly it can be seen that flagged spots within each of the 48 squares were distributed in the upper third of the slide. Control and hypothetical genes are printed in the upper third of each grid.

Figure 30 - Spots in red are seen as straight lines at the top of each grid. They represent landing lights and the scorecard.

Figure 29 shows the QC image when the flag value (-75) was used to identify the landing lights and scorecard spots. Landing lights were always seen as 4 green spots in the corner of each grid. They were used to guide the gal file to line up each spot with its representative information (gene name and position on the slide). The scorecard was a series of spots on the top row of each grid. The scorecard was the mixture of oligonucleotides added to the probe mix and show up as increasingly bright spots. They were used as a quality control measure to confirm that each Cye dye colour is correctly represented on the slide and it also provided a calibration scale.

III. Processes: Filters, Normalisation and visualisation

Using the “add process” button filtering parameters, normalisation and visualisation plots were added and edited (see figure 30).



Data | Process | Notes | Post Compilation

-	USE	Process Type	Parameters
✕	☑	String Filter	ID Equals pjt.*
✕	☑	String Filter	ID Equals ""
✕	☑	String Filter	ID Equals landing.*
✕	☑	String Filter	ID Equals arabidopsis.*
✕	☑	One Way Field Filter	Circularity <= 70.0
✕	☑	One Way Field Filter	Flags < 0.0
✕	☑	One Way Field Filter	F635 Median - B635 <= 0.0
✕	☑	One Way Field Filter	F532 Median - B532 <= 0.0
✕	☑	One Way Field Filter	% > B532+2SD < 80.0
✕	☑	One Way Field Filter	% > B635+2SD < 80.0
✕	☑	Value Filter	All values > 50000.0 for All channels
✕	☑	Value Filter	All values <= 100.0 for at least one channel
✕	☑	Global Lowess Normalization	A_VS_M , NoP=0.09 , WW=0.1 , IT=2
✕	☑	Spot Image View	No parameters
✕	☑	Plot	No parameters

Figure 31 - Processes and filters used and applied to all samples

IV. String Filters

String Filters identified and removed scorecard spots (pjt.*), unnamed spots, landing lights and arabidopsis (control) spots. Value filters were used to remove very low level intensity spots and a small number of (over)saturated spots. One Way Filters removed irregular spots (circularity), spots flagged by GenePix as "not found", spots where the foreground intensity was no different to the background intensity (F532 Median – B532<=0) for both channels. The one way filter %> B635+2SD <80, removed all spots where less than 80 % of the foreground pixels were above 2x the standard deviation of the background. Basically, this removed spots which had an intensity very close to the background intensity and therefore could not be differentiated. The figure of 80% was chosen as some of the arrays were noticed to have very high background level, and hence spots were being included in the data which had a signal intensity no different to the background. Increasing to 80% removed most of these spots.

Each stage was visualised using “Plot” to see the data that had been filtered - see figures 31-35.

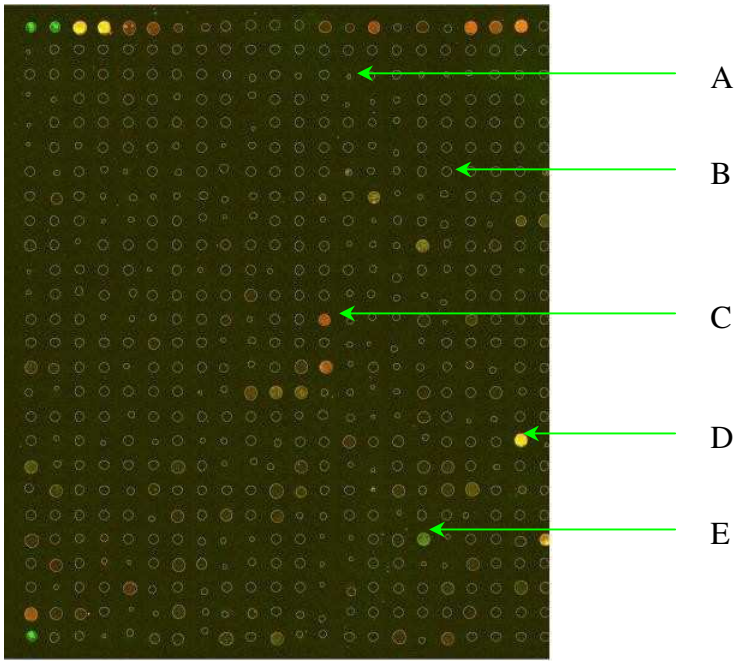


Figure 32 - Spotpix view showing the mask, i.e. all spots that have been detected in genepix. A= small spot, B = normal size hollow intensity spot, C = up-regulated spot, D = equally expressed spot, E= down-regulated spot.

Figure 31 demonstrates what Genepix sees as potential spots prior to filtering. It looked for all spots in the grid and highlighted them. The arrow labelled A points to an obviously small spot which we hoped to remove. It was not necessary to use a filter based on spot size as these spots were removed by the value filter <100 (see figure 34). Spot B was a spot of normal size, however, its intensity was no different to the overall background slide intensity and was removed by any of the latter 4 one way field filters shown in figure 30. Spot C was a spot that we were interested in and is shown in red. It represents a gene that was up-regulated in the sample compared to the control sample. Spot D is a spot that was expressed equally in the test versus control sample. Spot E is a spot that was down-regulated in the test versus control sample.

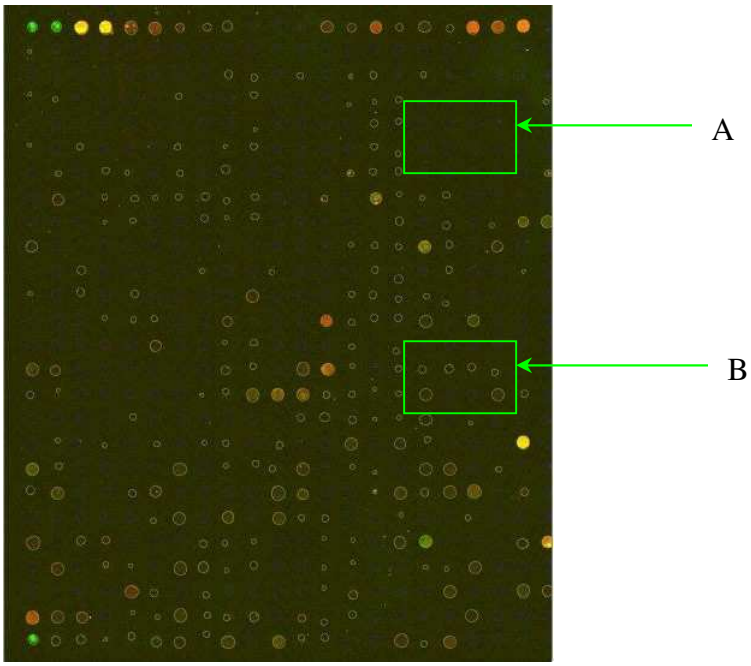


Figure 33 - Spotpix view demonstrating the remaining circled spots that are left following removal of spots flagged by genepix. Box A - spots filtered. Box B = unfiltered unwanted spots.

In figure 32, box A contains an area of the slide where all spots were filtered out. It can be seen that there are still a large number of small, irregular or low intensity spots that are close to the background. Box B demonstrates an area where all of the spots contained within it are unwanted. The value filter was used to remove spots where either one or both channel intensities was less than 100 and was effective in removing these spots, see figure 33. In practical terms this filter removed spots which had very low intensities that were no different to the slide background being neither positively nor negatively expressed in that sample and therefore of no interest. Other filter options could have been used to remove for example, small spots of less than 50 um (the average spots diameter being around 75 um). However, the intensity value filter proved to be effective alone.

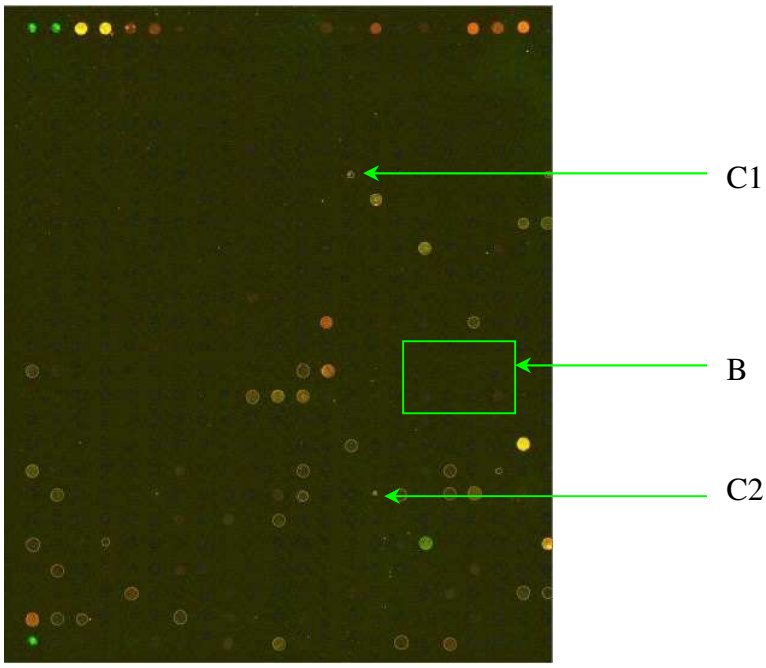


Figure 34 - Spotpix view highlighting only those spots that have been left following filtering stages suitable for further analysis. Box B = spots filtered by intensity value filter. C1 and C2 = remaining spots / artefact.

Figure 33 shows the same box B as shown in figure 32 following filtering. It can be seen that the unwanted spots were removed. It was noticed that there remained a few small spots labelled C1 and C2. It was not possible to remove every unwanted spot and artefact. However, only spots that had a faint light ring around them were recognised by genepix. Therefore, the remaining spots and artefacts were not included in the extracted data and so had no consequence on subsequent analysis.

The histogram in figure 34 demonstrates pre-normalised data that had been filtered using the processes shown in Figure 30. The plot is log10 foreground - background intensity (median value of pixels making up a spot) of 532nm green channel vs. log10 foreground - background intensity of the 635 nm (red) channel. Each dot represents a gene.

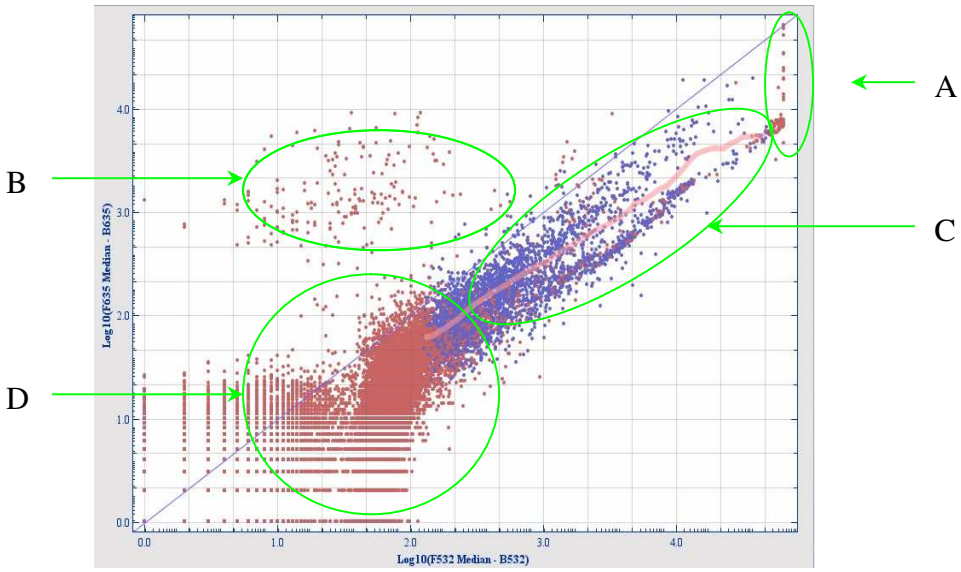


Figure 35 - Plot view highlighting pre-normalised filtered data (violet spots).
Filtered (out) spots are shown in red. The plot is of the difference between foreground and background intensity at 532 nm vs. the difference between foreground and background intensities in the 635 nm channel.
A = Marginal spots, B = landing lights, scorecard, C = Filtered data, D = Filtered out data

In Figure 34 the group of spots encircled A were saturated spots that would potentially skew the data if included in the dataset and were removed by the intensity value filter. Group B contains landing lights and scorecard spots. Group C contains filtered data that was used in subsequent analysis. The line of best fit shown in red can be seen to be below x = y. The data was therefore seen to be skewed. This occurred due to the variability in hybridisation across the experiments. The plot was the difference between foreground and background intensity at 532 nm vs. the difference between foreground and background intensities in the 635 nm channel. If one channel's background e.g. 532nm predominated the overall appearance of the slide would have been green. In order to correct for these differences so that spot intensities were shown in proportion to the background, Lowess Global Normalisation was performed.

V. Lowess Global Normalisation (locally weighted scatter plot smoothing)

This normalisation technique was used to correct for differences in dye binding, background and image scanner settings that potentially affected dye intensity measurements [150]. Should the discrepancies not be corrected then this was likely to lead to the data being unfit for analysis. The histogram in Figure 35 shows the filtered data shown in Figure 34 following Lowess Normalisation.

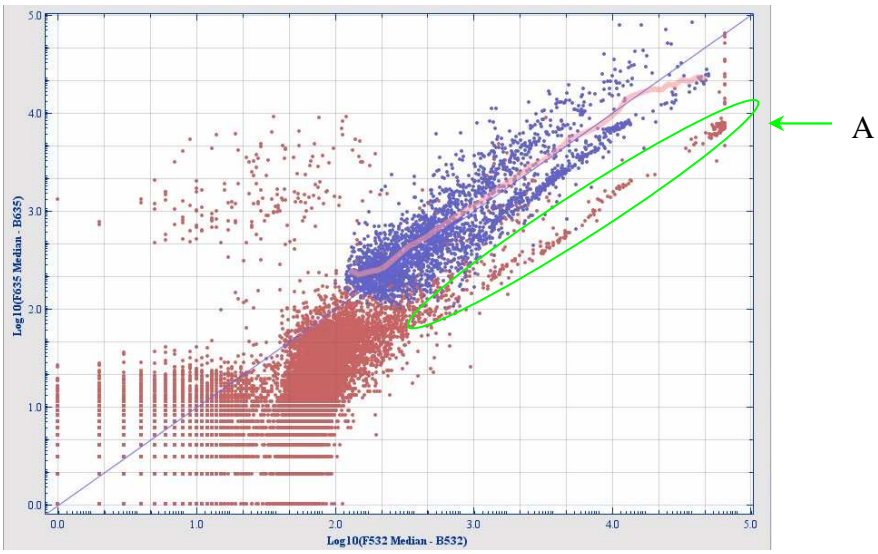


Figure 36 - Plot view highlighting normalised filtered data (violet spots). Additional spots are seen to be filtered out following normalisation.

The data can now be seen to be shifted in order to correct for the above mentioned potential discrepancies that would otherwise lead to inaccurate analysis. Interestingly additional spots labelled A are seen to have been filtered out that were not visible in the pre-normalised dataset.

3.4 Data Analysis

Sample numbers were a limiting factor in the overall scope of analysis. The above mentioned analyses were performed on the following groups of samples (scenarios).

A. Deceased Donors (Immediate function) versus Living Donors (Immediate function).
B. Deceased Donors (Immediate function) vs. all Non-immediately functioning donor kidneys (Moderate / Poor / Non-function)
C. Deceased Donors (Immediate function) vs. Deceased donors: moderate / poor /non-function

3.4.1 Deceased Donors (Immediate function) versus Living Donors (Immediate function).

The purpose of this analysis was to identify genes to highlight differences in the two groups that were as a result of brain-stem death (autonomic storm) and prolonged cold ischaemia in the deceased donor kidney compared to the living donor kidney.

3.4.1.1 Significance Analysis of Microarrays.

Gene expression in the two groups was compared using SAM which allows genes which are significantly up or down-regulated in the test groups compared to the control group to be identified with a false discovery rate of 5%.

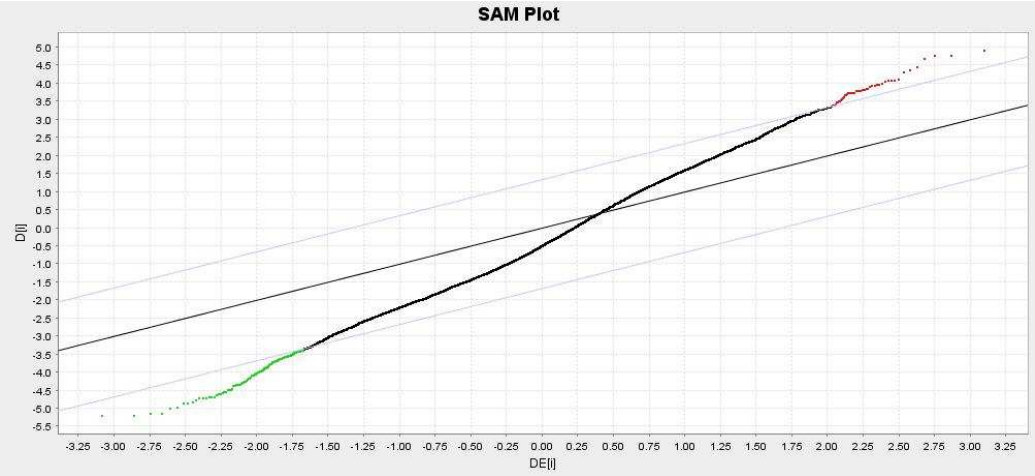


Figure 37 - SAM graph for genes differentially expressed in the Deceased donor compared to the Living Donor samples, (False discovery rate = 0 and delta = 1.375). Green dots represent down-regulated genes and red dots up-regulated genes in the deceased versus living donor dataset (with respect to the control sample).

In Figure 36 the two faint lines represent the region in which genes have not been significantly differentially expressed at the chosen FDR 5%. The red dots shows those genes that are significantly up-regulated and the green genes show significantly down-regulated genes (with respect to the control sample). The genes whose plot values are represented in black are considered non-significant at the FDR of 5%. The same control sample was used for each sample so we

assume that differentially expressed genes in one deceased donor group are up or down-regulated in comparison to the living donor group. Genes that were expressed equally are not shown or analysed further.

Using a false discovery rate FDR of 5% identified 1802 genes. An FDR of 1% identified 606 genes and an FDR of 0% (delta = 1.375) 190 genes. The following analyses were performed using the larger dataset (1802 genes), in order to prevent the analysis from becoming too narrowed and in the case of gene ontology and pathway analysis; to allow a wider diversity of system and pathways to be identified. The following list of genes identified by SAM and the hierarchical clustering heatmap are based on the 190 genes (FDR 0%) dataset.

Table 12 contains the list of genes identified by SAM (FDR 0%) that were expressed at higher levels in the deceased group versus the living donor group. SAM detected 190 genes, 143 were down-regulated and 47 were up-regulated. The fold change refers to the amount of expression in the first set of samples compared to the expression in the second, e.g. for NM003810 (tumour necrosis factor (ligand)) expression in the deceased donor group was 81.68 times more than in the living donor group.

Genbank ID	Description	Fold Change
NM_003810	Homo sapiens tumour necrosis factor (ligand) super family, member 10 (TNFSF10), mRNA.	81.68
NM_021983	Homo sapiens major histocompatibility complex, class II, DR beta 4 (HLA-DRB4), mRNA	23.44
NM_005851	DOC-1 related protein (DOC-1R).	22.1
NM_002668	Intestinal membrane A4 protein (Proteolipid protein 2).	14.68
NM_003651	Homo sapiens cold shock domain protein A (CSDA), mRNA	12.31
NM_017867	Homo sapiens chromosome 4 open reading frame 27 (C4orf27), mRNA	8.13
NM_018147	Fas apoptotic inhibitory molecule 1.	7.63
NM_005567	Galectin-3 binding protein precursor (Lectin galactoside-binding soluble 3 binding protein) (Mac-2 binding protein) (Mac-2 BP) (MAC2BP) (Tumour-associated antigen 90K).	7.53

NM_004748	Homo sapiens cell cycle progression 1 (CCPG1), transcript variant 1, mRNA	7.52
NM_144990	Homo sapiens schlafen-like 1 (SLFNL1), mRNA	7.26
NM_030922	Homo sapiens non imprinted in Prader-Willi/Angelman syndrome 2 (NIPA2), transcript variant 1, mRNA	7.09
NM_006276	Splicing factor, arginine/serine-rich 7 (Splicing factor 9G8). [Source:Uniprot/SWISSPROT;Acc:Q16629]	7.04
NM_022350	leukocyte-derived arginine aminopeptidase [Source:RefSeq_peptide;Acc:NP_071745]	6.84
NM_002027	Protein farnesyltransferase/geranylgeranyltransferase type I alpha subunit (EC 2.5.1.58) (EC 2.5.1.59) (CAAX farnesyltransferase alpha subunit)	6.19
NM_006947	Homo sapiens signal recognition particle 72kDa (SRP72), mRNA	6.08
NM_006708	Lactoylglutathione lyase (EC 4.4.1.5) (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I) (Glx I) (Ketone-aldehyde mutase) (S-D- lactoylglutathione methylglyoxal lyase).	5.13
NM_003342	Ubiquitin-conjugating enzyme E2 G1 (EC 6.3.2.19) (Ubiquitin-protein ligase G1) (Ubiquitin carrier protein G1) (E217K) (UBC7). [Source:Uniprot/SWISSPROT;Acc:P62253]	5.13
NM_001797	Cadherin-11 precursor (Osteoblast-cadherin) (OB-cadherin) (OSF-4). [Source:Uniprot/SWISSPROT;Acc:P55287]	5.07
NM_032905	Splicing factor 45 (45kDa splicing factor) (RNA binding motif protein 17). [Source:Uniprot/SWISSPROT;Acc:Q96I25]	5.04
NM_014740	Probable ATP-dependent helicase DDX48 (DEAD-box protein 48) (Eukaryotic initiation factor 4A-like NUK-34) (Nuclear matrix protein 265) (hNMP 265) (Eukaryotic translation initiation factor 4A isoform 3).	4.99
NM_002571	Glycodelin precursor (GD)	4.82
NM_025207	FAD synthetase isoform 2 [Source:RefSeq_peptide;Acc:NP_958800]	4.79
NM_024618	Homo sapiens NLR family member X1 (NLRX1), transcript variant 1, mRNA	4.75
NM_001788	Septin 7 (CDC10 protein homolog). [Source:Uniprot/SWISSPROT;Acc:Q16181]	4.71
NM_021628	Epidermis-type lipoxygenase 3 (EC 1.13.11.-) (e-LOX-3). [Source:Uniprot/SWISSPROT;Acc:Q9BYJ1]	4.66
NM_000173	Homo sapiens glycoprotein Ib (platelet), alpha polypeptide (GP1BA), mRNA	4.65
NM_006848	Delta-interacting protein A (Hepatitis delta antigen interacting protein A). [Source:Uniprot/SWISSPROT;Acc:Q15834]	4.17
NM_030967	Homo sapiens keratin associated protein 1-1 (KRTAP1-1), mRNABottom of Form	4.13
NM_006931	Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA	4.05
NM_020401	Nuclear pore complex protein Nup107 (Nucleoporin Nup107) (107 kDa nucleoporin). [Source:Uniprot/SWISSPROT;Acc:P57740]	4.03
NM_002576	Serine/threonine-protein kinase PAK 1 (EC 2.7.1.37) (p21-activated kinase 1) (PAK-1) (P65-PAK) (Alpha-PAK). [Source:Uniprot/SWISSPROT;Acc:Q13153]	3.99

NM_002932	Homo sapiens regulator of mitotic spindle assembly 1 (RMSA1), mRNA	3.82
NM_002663	Phospholipase D2 (EC 3.1.4.4) (PLD 2) (Choline phosphatase 2) (Phosphatidylcholine-hydrolyzing phospholipase D2) (PLD1C) (hPLD2). [Source:Uniprot/SWISSPROT;Acc:O14939]	3.81
NM_001345	Diacylglycerol kinase, alpha (EC 2.7.1.107) (Diglyceride kinase) (DGK- alpha) (DAG kinase alpha) (80 kDa diacylglycerol kinase). [Source:Uniprot/SWISSPROT;Acc:P23743]	3.71
NM_000458	Hepatocyte nuclear factor 1-beta (HNF-1beta) (HNF-1B) (Variant hepatic nuclear factor 1) (VHNF1) (Homeoprotein LFB3) (Transcription factor 2) (TCF-2).	3.67
NM_015660	GTPase, IMAP family member 2 (Immunity-associated protein 2) (hIMAP2). [Source:Uniprot/SWISSPROT;Acc:Q9UG22]	3.61
NM_014433	Rhabdoid tumour deletion region protein 1. [Source:Uniprot/SWISSPROT;Acc:Q9UHP6]	3.55
NM_000434	Sialidase 1 precursor (EC 3.2.1.18) (Lysosomal sialidase) (N-acetyl- alpha-neuraminidase 1) (Acetylneuraminyl hydrolase) (G9 sialidase).	3.4
NM_014916	Homo sapiens lemur tyrosine kinase 2 (LMTK2), mRNA	3.21
NM_012127	Cip1-interacting zinc finger protein (Nuclear protein NP94). [Source:Uniprot/SWISSPROT;Acc:Q9ULV3]	3.12
NM_021216	Endothelial zinc finger protein induced by tumour necrosis factor alpha (Zinc finger protein 71) (ZNF47). [Source:Uniprot/SWISSPROT;Acc:Q9NQZ8]	2.95
NM_020298	Sulfonylurea receptor 2. [Source:Uniprot/SWISSPROT;Acc:O60706]	2.91
NM_005215	Netrin receptor DCC precursor (Tumour suppressor protein DCC) (Colorectal cancer suppressor). [Source:Uniprot/SWISSPROT;Acc:P43146]	2.78
NM_005381	Nucleolin (Protein C23). [Source:Uniprot/SWISSPROT;Acc:P19338]	2.37
NM_003991	Homo sapiens endothelin receptor type B (EDNRB), transcript variant 2, mRNA	2.19

Table 12 - Table of - genes expressed at a higher level identified by SAM. FDR = 0% Deceased donors versus Living donors. The right hand column indicates the fold change; the gene is up-regulated in the deceased group in comparison to the living donor group by x fold. Only 45 genes are shown as the other two are hypothetical genes

Two genes NM 021983 and NM 0038 were seen to up-regulated by 23 and 82 times in the deceased donor group compared to the living donor group.

Table 13 contains the list of genes expressed at a lower level, identified by SAM (FDR 0%) that were in the deceased group compared to the living donor group.

GenBank ID	Description	Fold Change
nm_024774	glutamine and serine rich 1 (predicted)	-235.423
nm_004646	nephrosis 1 homolog, nephrin (human)	-160.492
nm_001254	cell division cycle 6 homolog (S. cerevisiae) (predicted)	-92.774
nm_016827	8-oxoguanine DNA-glycosylase 1	-89.555
nm_032630	similar to cyclin-dependent kinase 2-interacting protein (predicted)	-72.186
nm_001487	biogenesis of lysosome-related organelles complex-1, subunit 1 (predicted)	-71.208
nm_006032	similar to Copine-6 (Copine VI) (Neuronal-copine) (N-copine)	-61.66
nm_004003	carnitine acetyltransferase	-41.927
nm_024811	similar to RIKEN cDNA 5730453I16	-41.127
nm_019899	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	-39.396
nm_003220	transcription factor AP-2, alpha (predicted)	-38.949
xm_049237	RIKEN cDNA 2310022K01 gene	-36.527
af118078	PRO1848 protein	-34.702
nm_032592	similar to 2610203E10Rik protein (predicted)	-32.526
nm_007372	DEAD (Asp-Glu-Ala-Asp) box polypeptide 42 (predicted)	-32.24
nm_016292	TNF receptor-associated protein 1	-31.553
nm_002027	farnesyltransferase, CAAX box, alpha	-29.756
nm_012127	CDKN1A interacting zinc finger protein 1 (predicted)	-29.736
nm_025146	Mak3 homolog (S. cerevisiae) (predicted)	-27.593
nm_003849	succinate-CoA ligase, GDP-forming, alpha subunit	-26.52
nm_012088	6-phosphogluconolactonase (predicted)	-26.035
nm_016627	similar to X83328 protein	-25.334
nm_017984	zinc finger, CW-type with PWWP domain 1 (predicted)	-23.386
nm_023948	motile sperm domain containing 3	-22.682
nm_033058	ring finger protein 29	-22.328
nm_003991	endothelin receptor type B	-21.214
nm_002273	keratin complex 2, basic, gene 8	-19.263
nm_004493	hydroxyacyl-Coenzyme A dehydrogenase type II	-19.234
nm_018457	similar to RIKEN cDNA 1110020C13	-17.08
nm_015660	GTPase, IMAP family member 2	-15.793

nm_000789	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	-15.622
nm_004551	NADH dehydrogenase (ubiquinone) Fe-S protein 3 (predicted)	-15.236
bc012493	similar to RIKEN cDNA E330009J07 gene (predicted)	-15.007
nm_006003	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	-14.533
nm_033640	SCAN domain containing 2	-13.666
nm_001797	cadherin 11	-13.309
nm_005866	opioid receptor, sigma 1	-12.431
nm_032383	Hermansky-Pudlak syndrome 3 homolog (human) (predicted)	-11.978
nm_020549	choline acetyltransferase (predicted)	-11.963
nm_005215	deleted in colorectal carcinoma	-11.326
nm_020979	adaptor protein with pleckstrin homology and src homology 2 domains	-11.128
nm_014916	similar to lemur tyrosine kinase 2	-10.748
nm_024029	Yip1 domain family, member 2	-10.573
nm_014138	transmembrane protein 29	-10.469
nm_005809	peroxiredoxin 2	-10.325
nm_016730	folate receptor 1 (adult)	-10.303
nm_032445	similar to MEGF11 protein	-10.252
nm_013945	similar to paired box gene 7 isoform 1 (predicted)	-10.115
nm_018355	zinc finger protein 415	-10.089
nm_005851	similar to DOC-1 related protein	-10.025
nm_044472	cell division cycle 42 homolog (S. cerevisiae)	-9.792
nm_005083	U2 small nuclear RNA auxillary factor 1-like 1	-9.669
nm_003260	transducin-like enhancer of split 2, homolog of Drosophila E(spl)	-9.343
nm_001859	solute carrier family 31 (copper transporters), member 1	-9.334
nm_003611	similar to Ofd1 protein (predicted)	-8.95
nm_020630	ret proto-oncogene	-8.828
nm_033297	NACHT, leucine rich repeat and PYD containing 12 (predicted)	-8.448
nm_000281	pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 1	-8.398
nm_014960	arylsulfatase G	-8.384
nm_012228	methionine sulfoxide reductase B2	-8.365
nm_020552	T-cell leukemia/lymphoma 6	-8.308
nm_018838	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12 (predicted)	-8.15
nm_006848	similar to Delta-interacting protein A (Hepatitis delta antigen interacting protein A) (predicted)	-7.932

nm_014232	vesicle-associated membrane protein 2	-7.856
nm_005013	nucleobindin 2	-7.854
nm_002446	mitogen activated protein kinase 10	-7.751
nm_002436	membrane protein, palmitoylated	-7.557
nm_018060	isoleucine-tRNA synthetase 2, mitochondrial (predicted)	-7.086
nm_001152	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	-6.796
nm_004905	peroxiredoxin 6	-6.778
nm_018249	CDK5 regulatory subunit associated protein 2	-6.705
nm_001183	ATPase, H+ transporting, lysosomal accessory protein 1	-6.581
nm_003776	mitochondrial ribosomal protein L40	-6.537
nm_003685	KH-type splicing regulatory protein	-6.531
nm_004548	similar to NADH-ubiquinone oxidoreductase PDSW subunit (Complex I-PDSW) (CI-PDSW)	-6.511
nm_018237	similar to cell division cycle and apoptosis regulator 1 (predicted)	-6.347
nm_003334	similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	-6.071
nm_020414	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	-6.067
nm_144990	similar to hypothetical protein 4933406A14	-5.778
nm_013996	tachykinin 1	-5.724
nm_024308	similar to Hypothetical protein MGC18716	-5.661
nm_003477	hypothetical protein LOC311254	-5.554
nm_005601	natural killer cell group 7 sequence	-5.42
nm_025107	myc target 1 (predicted)	-5.381
nm_002931	ring finger protein 1	-5.315
nm_000528	mannosidase 2, alpha B1	-5.287
l38593	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	-5.283
nm_022785	similar to RIKEN cDNA 4931407K02 (predicted)	-5.237
nm_006774	similar to indolethylamine N-methyltransferase	-5.194
af258584	similar to hypothetical protein FLJ20003 (predicted)	-5.125
nm_005918	malate dehydrogenase, mitochondrial	-5.121
nm_022805	small nuclear ribonucleoprotein N	-5.038
nm_016504	mitochondrial ribosomal protein L27 (predicted)	-5.031
nm_020677	similar to RIKEN cDNA 1110025F24 (predicted)	-4.841
nm_003969	ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast) (predicted)	-4.798
nm_021947	serine racemase	-4.721
nm_004568	serine (or cysteine) peptidase inhibitor, clade B, member 6a	-4.72

nm_002051	GATA binding protein 3	-4.689
nm_022095	zinc finger protein 335	-4.683
nm_006012	similar to Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial precursor (Endopeptidase Clp)	-4.521
nm_004870	mannose-P-dolichol utilization defect 1	-4.484
nm_014940	MON1 homolog b (yeast) (predicted)	-4.46
nm_133371	similar to myozenin 3	-4.253
nm_000086	ceroid lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease)	-4.236
nm_024622	similar to RIKEN cDNA 5330408N05 gene (predicted)	-4.081
nm_005956	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase	-4.071
nm_000383	autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) (predicted)	-4.059
nm_002501	nuclear factor I/X	-4.053
nm_013325	ATG4 autophagy related 4 homolog B (S. cerevisiae)	-4.006
nm_000121	erythropoietin receptor	-3.994
nm_057092	FK506 binding protein 2 (predicted)	-3.914
nm_032261	similar to Putative protein C21orf56 homolog	-3.846
nm_003975	SH2 domain protein 2A	-3.836
nm_021979	heat shock 70kDa protein 2	-3.803
nm_024051	similar to C44B7.7	-3.736
nm_015492	similar to DKFZP434H132 protein	-3.633
nm_003950	coagulation factor II (thrombin) receptor-like 3	-3.533
nm_000297	similar to polycystic kidney disease 2 (predicted)	-3.499
nm_004360	cadherin 1	-3.49
nm_000173	similar to glycoprotein 1b, alpha polypeptide	-3.276
nm_006708	glyoxylase 1	-2.962
nm_022459	exportin 4 (predicted)	-2.764
nm_014433	rhabdoid tumor deletion region gene 1	-2.654

Table 13 - Table of - genes expressed at a lower level identified by SAM. FDR = 0%. Deceased donors versus Living donors. The right hand column indicates the fold change; the gene is down-regulated in the deceased group in comparison to the living donor group by x fold.

In tables 12 and 13 more genes are shown to be down-regulated in the deceased versus living donor group, with a greater fold change in expression values.

3.4.1.2 Hierarchical clustering

In order to visualise the data shown in tables 12 and 13 and to potentially differentiate trends of up or down-regulation in genes across samples or groups of samples, hierarchical clustering was performed on the dataset deceased vs. living donor (0% FDR). Hierarchical clustering was performed in J-Express using WPGMA (weighted pair group method with arithmetic mean) [145]. In order to potentially highlight groups of genes that were only found in one or other group (e.g. deceased donors), samples were also included in the clustering algorithm.

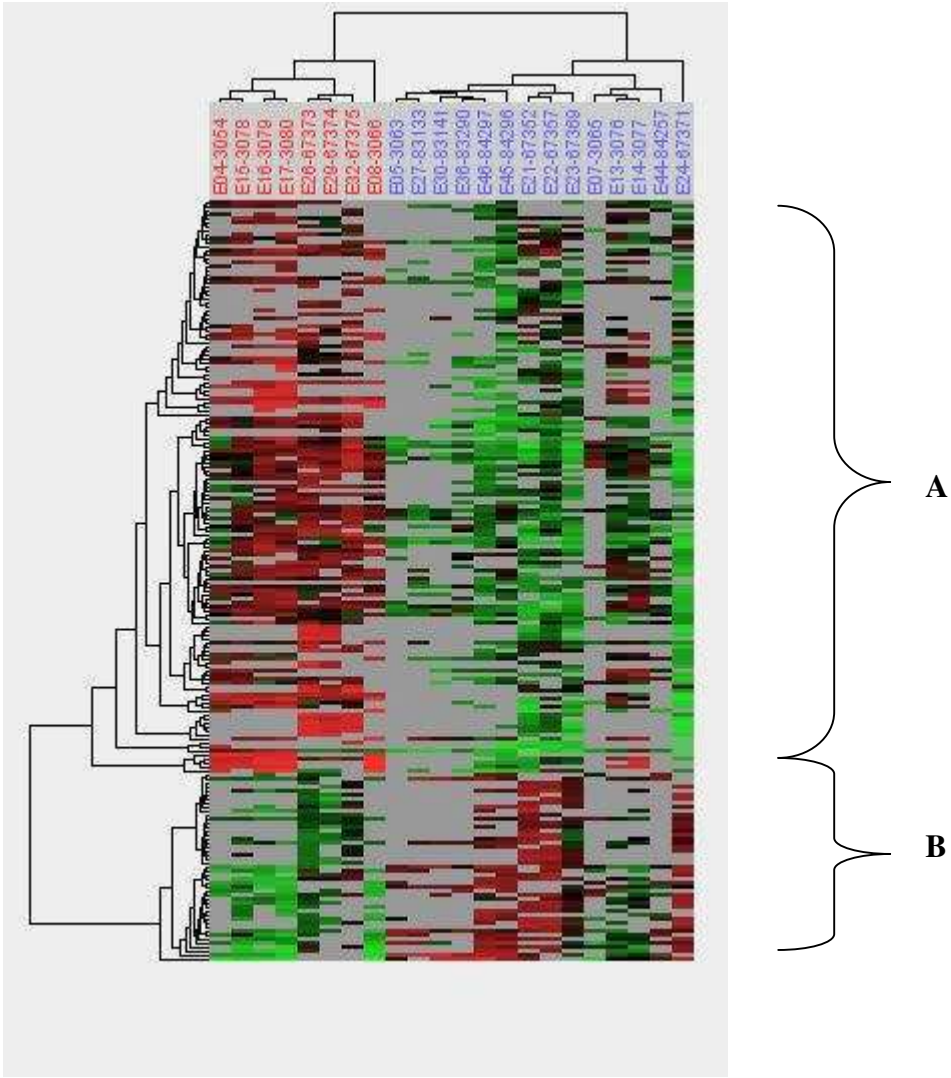


Figure 38 Hierarchical clustering of significantly differentially expressed genes identified by SAM (FDR 0%). Red column labels represent living donors and blue column labels represent deceased donors. . Group A refers to genes that were down-regulated in the deceased versus living donor group. Group B refers to genes that were up-regulated in the deceased donors group (compared to the living donor group)

In figure 37 hierarchical clustering reveals two main groups of genes labelled A and B. Cluster A represents genes that were down-regulated in the deceased donor group compared to the living group. Group B represents genes that were up-regulated in the deceased versus living donor group.

a) Hierarchical clustering: up-regulated genes

Table 14 gives the list of genes that were upregulated in the deceased donor group.

Accession	Description
NM_015660	GTPase, IMAP family member 2 (Immunity-associated protein 2) (hIMAP2). [Source:Uniprot/SWISSPROT;Acc:Q9UG22]
NM_020298	Sulfonylurea receptor 2. [Source:Uniprot/SWISSPROT;Acc:O60706]
NM_005381	Nucleolin (Protein C23). [Source:Uniprot/SWISSPROT;Acc:P19338]
NM_012127	Cip1-interacting zinc finger protein (Nuclear protein NP94). [Source:Uniprot/SWISSPROT;Acc:Q9ULV3]
NM_021216	Endothelial zinc finger protein induced by tumor necrosis factor alpha (Zinc finger protein 71) (ZNF47). [Source:Uniprot/SWISSPROT;Acc:Q9NQZ8]
NM_005215	Netrin receptor DCC precursor (Tumor suppressor protein DCC) (Colorectal cancer suppressor). [Source:Uniprot/SWISSPROT;Acc:P43146]
NM_014433	Rhabdoid tumor deletion region protein 1. [Source:Uniprot/SWISSPROT;Acc:Q9UHP6]
NM_002571	Glycodelin precursor (GD) (Pregnancy-associated endometrial alpha-2 globulin) (PEG) (PAEG) (Placental protein 14) (Progesterone-associated endometrial protein) (Progestagen-associated endometrial protein). [Source:Uniprot/SWISSPROT;Acc:P09466]
NM_025207	FAD synthetase isoform 2 [Source:RefSeq_peptide;Acc:NP_958800]
NM_002663	Phospholipase D2 (EC 3.1.4.4) (PLD 2) (Choline phosphatase 2) (Phosphatidylcholine-hydrolyzing phospholipase D2) (PLD1C) (hPLD2). [Source:Uniprot/SWISSPROT;Acc:O14939]
NM_006848	Delta-interacting protein A (Hepatitis delta antigen interacting protein A). [Source:Uniprot/SWISSPROT;Acc:Q15834]
NM_021628	Epidermis-type lipoxygenase 3 (EC 1.13.11.-) (e-LOX-3). [Source:Uniprot/SWISSPROT;Acc:Q9BYJ1]
NM_002576	Serine/threonine-protein kinase PAK 1 (EC 2.7.1.37) (p21-activated kinase 1) (PAK-1) (P65-PAK) (Alpha-PAK). [Source:Uniprot/SWISSPROT;Acc:Q13153]
NM_001345	Diacylglycerol kinase, alpha (EC 2.7.1.107) (Diglyceride kinase) (DGK-alpha) (DAG kinase alpha) (80 kDa diacylglycerol kinase). [Source:Uniprot/SWISSPROT;Acc:P23743]
NM_001788	Septin 7 (CDC10 protein homolog). [Source:Uniprot/SWISSPROT;Acc:Q16181]

NM_006708	Lactoylglutathione lyase (EC 4.4.1.5) (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I) (Glx I) (Ketone-aldehyde mutase) (S-D- lactoylglutathione methylglyoxal lyase). [Source:Uniprot/SWISSPROT;Acc:Q04760]
NM_000458	Hepatocyte nuclear factor 1-beta (HNF-1beta) (HNF-1B) (Variant hepatic nuclear factor 1) (VHNF1) (Homeoprotein LFB3) (Transcription factor 2) (TCF-2). [Source:Uniprot/SWISSPROT;Acc:P35680]
NM_002668	Intestinal membrane A4 protein (Differentiation-dependent protein A4) (Proteolipid protein 2). [Source:Uniprot/SWISSPROT;Acc:Q04941]
NM_014740	Probable ATP-dependent helicase DDX48 (DEAD-box protein 48) (Eukaryotic initiation factor 4A-like NUK-34) (Nuclear matrix protein 265) (hNMP 265) (Eukaryotic translation initiation factor 4A isoform 3). [Source:Uniprot/SWISSPROT;Acc:P38919]
NM_000434	Sialidase 1 precursor (EC 3.2.1.18) (Lysosomal sialidase) (N-acetyl- alpha-neuraminidase 1) (Acetylneuraminyl hydrolase) (G9 sialidase).
NM_020401	Nuclear pore complex protein Nup107 (Nucleoporin Nup107) (107 kDa nucleoporin). [Source:Uniprot/SWISSPROT;Acc:P57740]
NM_003342	Ubiquitin-conjugating enzyme E2 G1 (EC 6.3.2.19) (Ubiquitin-protein ligase G1) (Ubiquitin carrier protein G1) (E217K) (UBC7). [Source:Uniprot/SWISSPROT;Acc:P62253]
NM_005567	Galectin-3 binding protein precursor (Lectin galactoside-binding soluble 3 binding protein) (Mac-2 binding protein) (Mac-2 BP) (MAC2BP) (Tumor-associated antigen 90K). [Source:Uniprot/SWISSPROT;Acc:Q08380]
NM_022350	leukocyte-derived arginine aminopeptidase [Source:RefSeq_peptide;Acc:NP_071745]
NM_006276	Splicing factor, arginine/serine-rich 7 (Splicing factor 9G8). [Source:Uniprot/SWISSPROT;Acc:Q16629]
NM_002027	Protein farnesyltransferase/geranylgeranyltransferase type I alpha subunit (EC 2.5.1.58) (EC 2.5.1.59) (CAAX farnesyltransferase alpha subunit) (Ras proteins prenyltransferase alpha) (FTase-alpha) (Type I protein geranyl-geranyltransferase alpha subunit) [Source:Uniprot/SWISSPROT;Acc:P49354]
NM_032905	Splicing factor 45 (45kDa splicing factor) (RNA binding motif protein 17). [Source:Uniprot/SWISSPROT;Acc:Q96I25]
NM_001797	Cadherin-11 precursor (Osteoblast-cadherin) (OB-cadherin) (OSF-4). [Source:Uniprot/SWISSPROT;Acc:P55287]
NM_018147	Fas apoptotic inhibitory molecule 1. [Source:Uniprot/SWISSPROT;Acc:Q9NVQ4]
NM_005851	DOC-1 related protein (DOC-1R). [Source:Uniprot/SWISSPROT;Acc:O75956]
NM_021983	HLA class II histocompatibility antigen, DR-W53 beta chain precursor. [Source:Uniprot/SWISSPROT;Acc:P13762]

Table 14 - Hierarchical clustering of significantly differentially expressed genes identified by SAM (FDR 0%) that were up-regulated in the deceased versus living donor group

b) Hierarchical clustering: down-regulated genes

Table 15 gives the list of genes that were downregulated in the deceased donor group.

Accession	Description
NM_000025	Beta-3 adrenergic receptor (Beta-3 adrenoceptor) (Beta-3 adrenoreceptor).
NM_000086	CLN3 protein (Battenin) (Batten disease protein).
NM_000121	Erythropoietin receptor precursor (EPO-R). [Source:Uniprot/SWISSPROT;Acc:P19235]
NM_000281	Pterin-4-alpha-carbinolamine dehydratase (EC 4.2.1.96) (PHS) (4-alpha-hydroxy-tetrahydropterin dehydratase) (Phenylalanine hydroxylase-stimulating protein) (Pterin carbinolamine dehydratase) (PCD) (Dimerization cofactor of hepatocyte nuclear factor 1-al
NM_000297	Polycystin 2 (Autosomal dominant polycystic kidney disease type II protein) (Polycystwin) (R48321). [Source:Uniprot/SWISSPROT;Acc:Q13563]
NM_000383	Autoimmune regulator (Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy protein) (APECED protein). [Source:Uniprot/SWISSPROT;Acc:O43918]
NM_000528	Lysosomal alpha-mannosidase precursor (EC 3.2.1.24) (Mannosidase, alpha B) (Lysosomal acid alpha-mannosidase) (Laman) (Mannosidase alpha class 2B member 1). [Source:Uniprot/SWISSPROT;Acc:O00754]
NM_001183	Vacuolar ATP synthase subunit S1 precursor (EC 3.6.3.14) (V-ATPase S1 subunit) (V-ATPase S1 accessory protein) (V-ATPase Ac45 subunit) (XAP-3). [Source:Uniprot/SWISSPROT;Acc:Q15904]
NM_001254	Cell division control protein 6 homolog (CDC6-related protein) (p62(cdc6)) (HsCDC6) (HsCDC18). [Source:Uniprot/SWISSPROT;Acc:Q99741]
NM_001398	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor (EC 5.3.3.-). [Source:Uniprot/SWISSPROT;Acc:Q13011]
NM_001487	Biogenesis of lysosome-related organelles complex-1, subunit 1 (BLOC-1 subunit 1) (GCN5-like protein 1) (RT14 protein). [Source:Uniprot/SWISSPROT;Acc:P78537]
NM_001626	RAC-beta serine/threonine-protein kinase (EC 2.7.1.37) (RAC-PK-beta) (Protein kinase Akt-2) (Protein kinase B, beta) (PKB beta). [Source:Uniprot/SWISSPROT;Acc:P31751]
NM_001859	High-affinity copper uptake protein 1 (hCTR1) (Copper transporter 1) (Solute carrier family 31, member 1). [Source:Uniprot/SWISSPROT;Acc:O15431]
NM_001963	Pro-epidermal growth factor precursor (EGF) [Contains: Epidermal growth factor (Urogastrone)]. [Source:Uniprot/SWISSPROT;Acc:P01133]
NM_002051	Trans-acting T-cell specific transcription factor GATA-3. [Source:Uniprot/SWISSPROT;Acc:P23771]
NM_002273	Keratin, type II cytoskeletal 8 (Cytokeratin 8) (K8) (CK 8). [Source:Uniprot/SWISSPROT;Acc:P05787]
NM_002436	55 kDa erythrocyte membrane protein (p55) (Membrane protein, palmitoylated 1). [Source:Uniprot/SWISSPROT;Acc:Q00013]
NM_002446	Mitogen-activated protein kinase 10 (EC 2.7.1.37) (Mixed lineage kinase 2) (Protein kinase MST). [Source:Uniprot/SWISSPROT;Acc:Q02779]
NM_002931	Polycomb complex protein RING1 (RING finger protein 1). [Source:Uniprot/SWISSPROT;Acc:Q06587]
NM_003220	Transcription factor AP-2 alpha (AP2-alpha) (Activating enhancer- binding protein 2 alpha) (AP-2 transcription factor) (Activator protein-2) (AP-2). [Source:Uniprot/SWISSPROT;Acc:P05549]

NM_003260	Transducin-like enhancer protein 2 (ESG2). [Source:Uniprot/SWISSPROT;Acc:Q04725]
NM_003477	Pyruvate dehydrogenase protein X component, mitochondrial precursor (Dihydrolipoamide dehydrogenase-binding protein of pyruvate dehydrogenase complex) (Lipoyl-containing pyruvate dehydrogenase complex component X) (E3-binding protein) (E3BP) (proX).
NM_003611	Oral-facial-digital syndrome 1 protein (Protein 71-7A). [Source:Uniprot/SWISSPROT;Acc:O75665]
NM_003685	Far upstream element binding protein 2 (FUSE binding protein 2) (KH type splicing regulatory protein) (KSRP) (p75). [Source:Uniprot/SWISSPROT;Acc:Q92945]
NM_003776	39S ribosomal protein L40, mitochondrial precursor (L40mt) (MRP-40) (Nuclear localization signal containing protein deleted in velocardiofacial syndrome) (Up-regulated in metastasis). [Source:Uniprot/SWISSPROT;Acc:Q9NQ50]
NM_003849	Succinyl-CoA ligase [GDP-forming] alpha-chain, mitochondrial precursor (EC 6.2.1.4) (Succinyl-CoA synthetase, alpha chain) (SCS-alpha). [Source:Uniprot/SWISSPROT;Acc:P53597]
NM_003975	SH2 domain protein 2A (T cell-specific adapter protein) (TSAd) (VEGF receptor-associated protein) (SH2 domain containing adapter protein). [Source:Uniprot/SWISSPROT;Acc:Q9NP31]
NM_004003	Carnitine O-acetyltransferase (EC 2.3.1.7) (Carnitine acetylase) (CAT) (Carnitine acetyltransferase) (CrAT). [Source:Uniprot/SWISSPROT;Acc:P43155]
NM_004360	Epithelial-cadherin precursor (E-cadherin) (Uvomorulin) (Cadherin-1) (CAM 120/80). [Source:Uniprot/SWISSPROT;Acc:P12830]
NM_004493	3-hydroxyacyl-CoA dehydrogenase type II (EC 1.1.1.35) (Type II HADH) (Endoplasmic reticulum-associated amyloid beta-peptide binding protein) (Short-chain type dehydrogenase/reductase XH98G2).
NM_004548	NADH-ubiquinone oxidoreductase PDSW subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-PDSW) (CI-PDSW). [Source:Uniprot/SWISSPROT;Acc:O96000]
NM_004551	NADH-ubiquinone oxidoreductase 30 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-30KD) (CI-30KD). [Source:Uniprot/SWISSPROT;Acc:O75489]
NM_004568	Placental thrombin inhibitor (Cytoplasmic antiproteinase) (CAP) (Protease inhibitor 6) (PI-6) (Serpin B6). [Source:Uniprot/SWISSPROT;Acc:P35237]
NM_004870	Mannose-P-dolichol utilization defect 1 protein (Suppressor of Lec15 and Lec35 glycosylation mutation homolog) (SL15). [Source:Uniprot/SWISSPROT;Acc:O75352]
NM_004905	Peroxiredoxin 6 (EC 1.11.1.-) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aiPLA2) (Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx) (24 kDa protein) (Liver 2D page spot 40 [Source:Uniprot/SWISSPROT;Acc:P30041]
NM_005326	Hydroxyacylglutathione hydrolase (EC 3.1.2.6) (Glyoxalase II) (GLX II). [Source:Uniprot/SWISSPROT;Acc:Q16775]

NM_005601	Protein NKG7 (Natural killer cell protein 7) (G-CSF-induced gene 1 protein) (Protein GIG-1). [Source:Uniprot/SWISSPROT;Acc:Q16617]
NM_005809	Peroxiredoxin 2 (EC 1.11.1.-) (Thioredoxin peroxidase 1) (Thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA) (PRP) (Natural killer cell enhancing factor B) (NKEF-B).
NM_005866	opioid receptor, sigma 1 isoform 3 [Source:RefSeq_peptide;Acc:NP_671514]
NM_005918	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37). [Source:Uniprot/SWISSPROT;Acc:P40926]
NM_005956	C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase) [Includes: Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5); Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9); Formyltetrahydrofolate synthetase (EC 6.3.4.3)].
NM_006012	Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial precursor (EC 3.4.21.92) (Endopeptidase Clp). [Source:Uniprot/SWISSPROT;Acc:Q16740]
NM_006032	Copine VI (Neuronal-copine) (N-copine). [Source:Uniprot/SWISSPROT;Acc:O95741]
NM_006774	Indolethylamine N-methyltransferase (EC 2.1.1.49) (Aromatic alkylamine N-methyltransferase) (Indolamine N-methyltransferase) (Arylamine N-methyltransferase) (Amine N-methyltransferase).
NM_007372	DEAD box polypeptide 42 protein [Source:RefSeq_peptide;Acc:NP_987095]
NM_012088	6-phosphogluconolactonase (EC 3.1.1.31) (6PGL). [Source:Uniprot/SWISSPROT;Acc:O95336]
NM_012228	Methionine-R-sulfoxide reductase B (EC 1.8.4.-) (CGI-131). [Source:Uniprot/SWISSPROT;Acc:Q9Y3D2]
NM_013247	Serine protease HTRA2, mitochondrial precursor (EC 3.4.21.-) (High temperature requirement protein A2) (HtrA2) (Omi stress-regulated endoprotease) (Serine proteinase OMI).
NM_013325	APG4 autophagy 4 homolog B isoform b [Source:RefSeq_peptide;Acc:NP_847896]
NM_013945	Paired box protein Pax-7 (HUP1). [Source:Uniprot/SWISSPROT;Acc:P23759]
NM_014138	transmembrane protein 29 [Source:RefSeq_peptide;Acc:NP_054857]
NM_014232	Vesicle-associated membrane protein 2 (VAMP-2) (Synaptobrevin 2). [Source:Uniprot/SWISSPROT;Acc:P63027]
NM_014940	HSV-1 stimulation-related 1 [Source:RefSeq_peptide;Acc:NP_055755]
NM_014960	Arylsulfatase G [Source:RefSeq_peptide;Acc:NP_055775]
NM_016292	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1). [Source:Uniprot/SWISSPROT;Acc:Q12931]
NM_018060	mitochondrial isoleucine tRNA synthetase [Source:RefSeq_peptide;Acc:NP_060530]
NM_018249	CDK5 regulatory subunit associated protein 2 (CDK5 activator-binding protein C48). [Source:Uniprot/SWISSPROT;Acc:Q96SN8]
NM_018355	zinc finger protein 415 [Source:RefSeq_peptide;Acc:NP_060825]

NM_018838	13kDa differentiation-associated protein [Source:RefSeq_peptide;Acc:NP_061326]
NM_020414	ATP-dependent RNA helicase DDX24 (DEAD-box protein 24). [Source:Uniprot/SWISSPROT;Acc:Q9GZR7]
NM_020677	HSCARG protein [Source:RefSeq_peptide;Acc:NP_065728]
NM_021947	Serine racemase (EC 5.1.1.-). [Source:Uniprot/SWISSPROT;Acc:Q9GZT4]
NM_021979	Heat shock-related 70 kDa protein 2 (Heat shock 70 kDa protein 2). [Source:Uniprot/SWISSPROT;Acc:P54652]
NM_022095	Zinc finger protein 335. [Source:Uniprot/SWISSPROT;Acc:Q9H4Z2]
NM_022785	CAP-binding protein complex interacting protein 1 isoform b [Source:RefSeq_peptide;Acc:NP_942153]
NM_023948	motile sperm domain containing 3 [Source:RefSeq_peptide;Acc:NP_076438]
NM_024811	pre-mRNA cleavage factor I, 59 kDa subunit
NM_025107	myc target 1 [Source:RefSeq_peptide;Acc:NP_079383]
NM_025146	Mak3 homolog [Source:RefSeq_peptide;Acc:NP_079422]
NM_032261	Putative protein C21orf56. [Source:Uniprot/SWISSPROT;Acc:Q9H0A9]
NM_032383	Hermansky-Pudlak syndrome 3 protein. [Source:Uniprot/SWISSPROT;Acc:Q969F9]
NM_032445	MEGF11 protein [Source:RefSeq_peptide;Acc:NP_115821]
NM_032592	1-aminocyclopropane-1-carboxylate synthase
NM_033640	SCAN domain-containing protein 2.
NM_133371	myozenin 3 [Source:RefSeq_peptide;Acc:NP_588612]
XM_034819	PREDICTED: zinc finger protein 629 [Source:RefSeq_peptide;Acc:XP_034819]
XM_049237	PREDICTED: KIAA0841 [Source:RefSeq_peptide;Acc:XP_049237]

Table 15 - Hierarchical clustering of significantly differentially expressed genes identified by SAM (FDR 0%) that were down-regulated in the deceased versus living donor group.

Groups A and B are analysed in more detail in the pathway analysis section.

3.4.1.3 Gene Ontology

a) Cellular processes

Gene Ontology mapping using the SAM dataset (FDR of 5%) identified the following cellular processes that were differentially expressed:

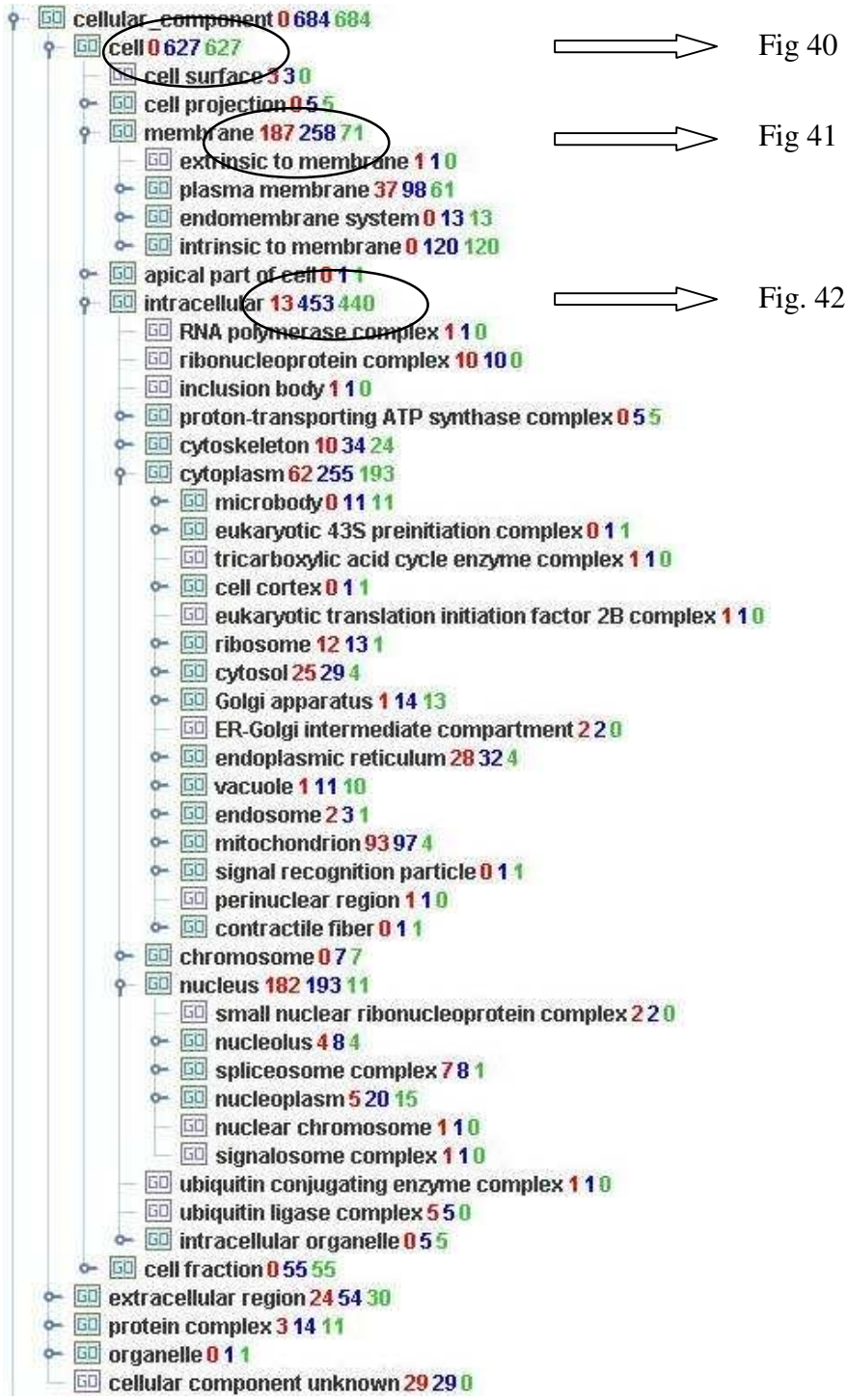


Figure 39 - The figure shows a GO-tree map of Cellular Processes based on deceased donor versus living donor (SAM FDR 5%) dataset

In figure 38 the red numbers in each term shows the number of genes in the dataset that correspond to the particular GO-term. The blue numbers represent the total number of genes corresponding to the GO-term and other terms downwards in the tree. The green number shows the difference between the blue and the red number, hence this is the number of genes annotated to nodes further down the line i.e. children nodes.

The following figures are representations of the go-terms (cell) membrane, intracellular and nucleus. For each term they show the breakdown into each sub-component of that term. It gives a quick visual representation of where the differentially expressed genes are situated.

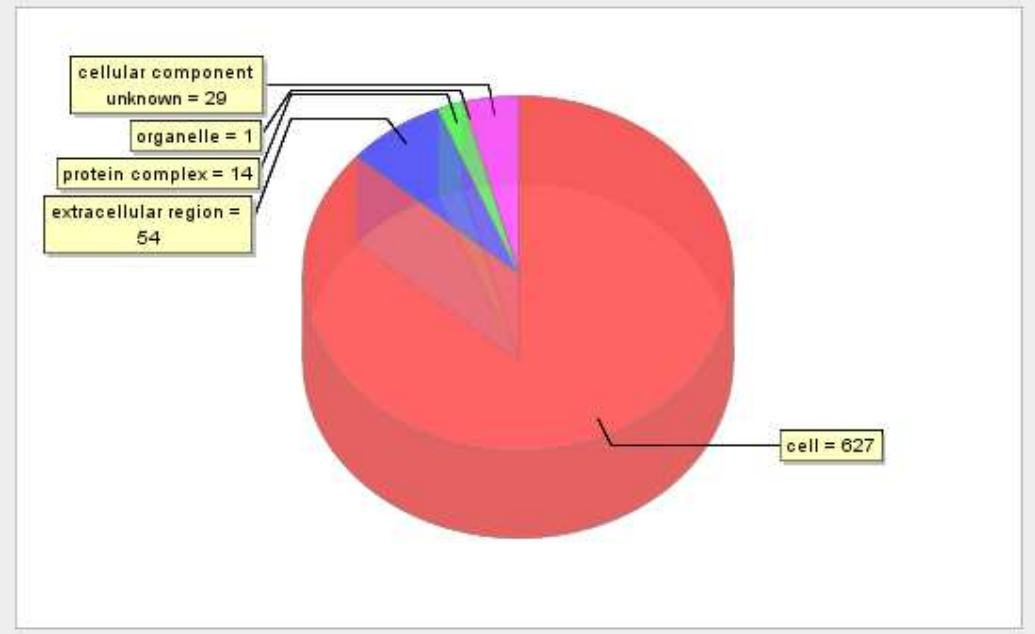


Figure 40 Cellular Components

Figure 39 depicts the number of cellular components that were differentially expressed. Within the term “cell” (627 components), shown in Figure 39 the sub-term “cellular membrane” is shown, in figure 40.

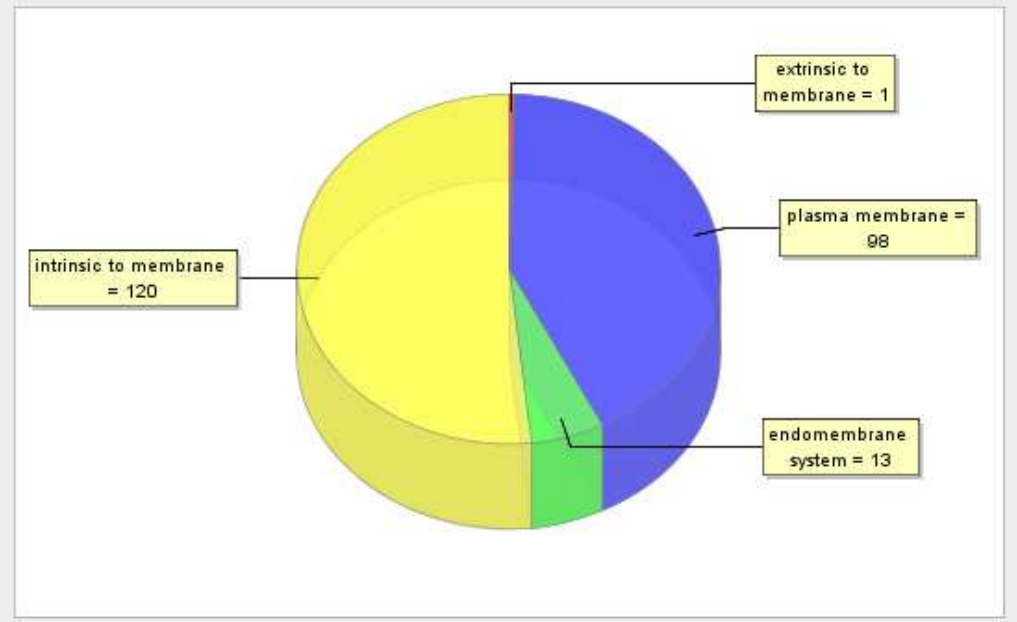


Figure 41 Processes involved the cell membrane

Most of the differentially expressed processes at the cell membrane level are shown in Figure 41 to be intrinsic to the cell membrane or plasma membrane. Figure 41 represents the intracellular processes that were differentially expressed within the cell membrane. Most processes are seen to involve the cytoplasm and nucleus.

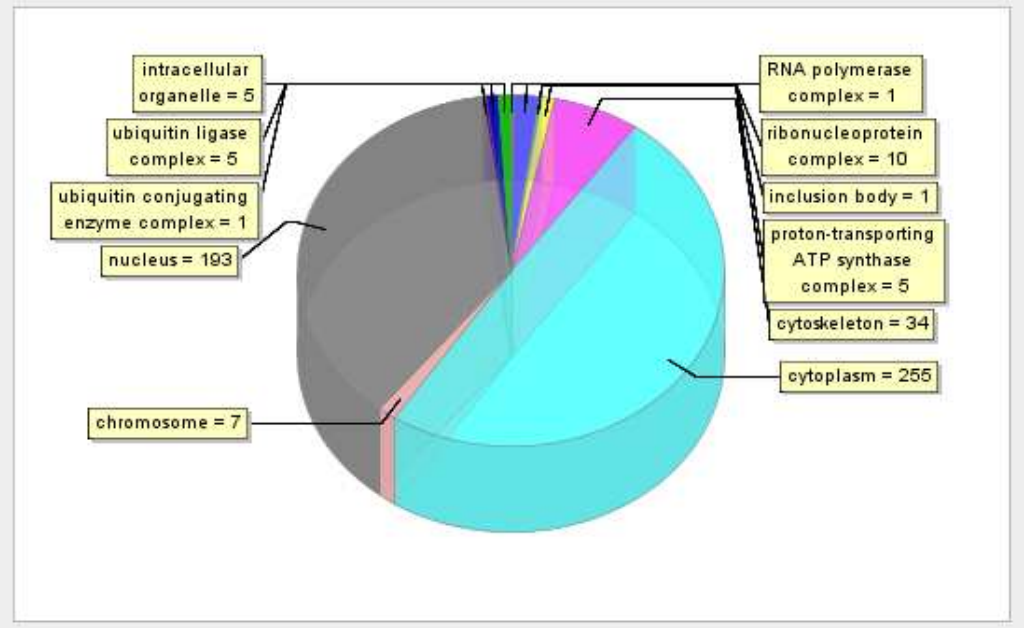


Figure 42 Intracellular processes

b) Molecular function

Gene Ontology mapping using the SAM dataset (FDR of 5%) identified the following molecular functions that were differentially expressed:

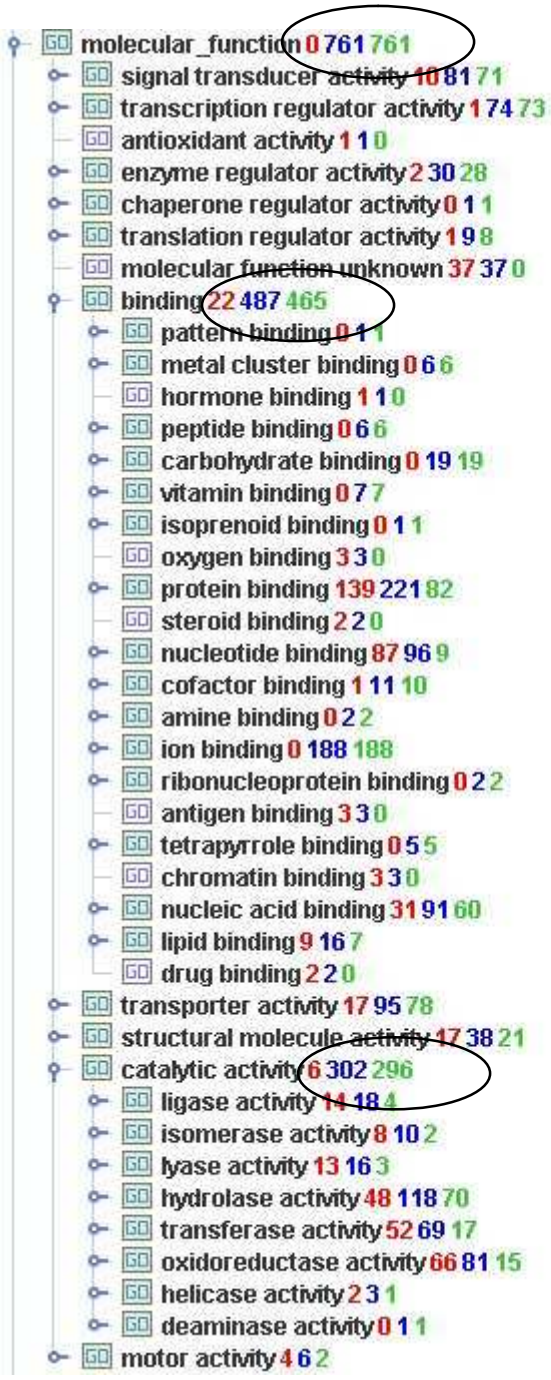


Fig 44

Fig 45

Fig 46

Figure 43 - GO-tree map of Molecular Function based on the deceased donor versus living donor (SAM FDR 5%) dataset

In Figure 44 the Go tree map for Molecular function is shown with its various components represented as a pie chart. Within molecular function the sub-terms binding and catalytic activity contained the majority of differentially expressed genes.

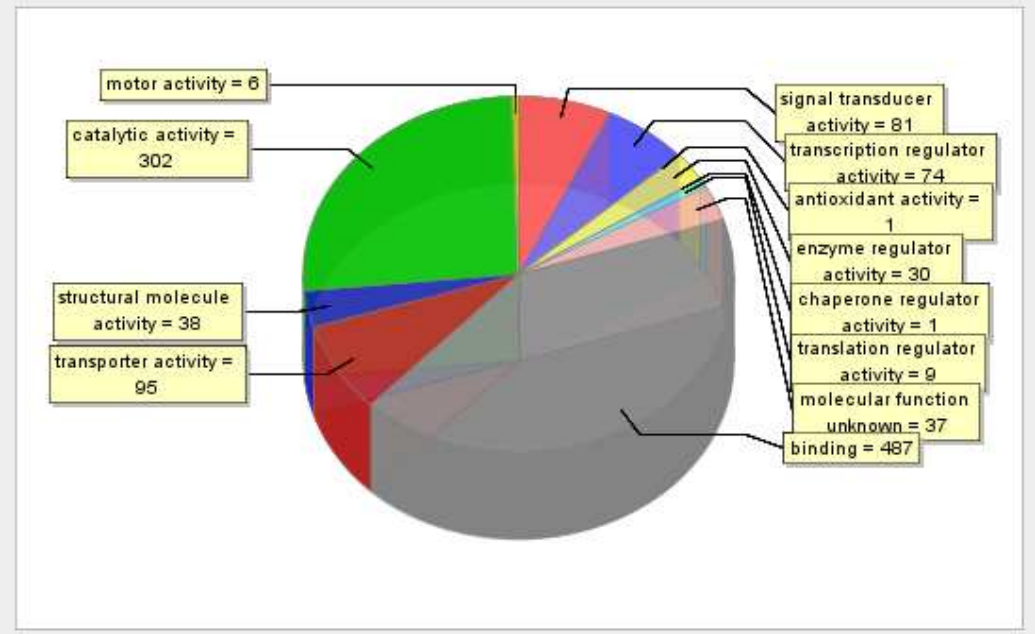


Figure 44 - Significantly differentially expressed genes involved in the term molecular functions

“Binding” is shown in grey in figure 43 and is broken down into its constituent components shown in figure 44. The processes protein binding, nucleotide binding and ion binding contain the majority of differentially expressed processes.

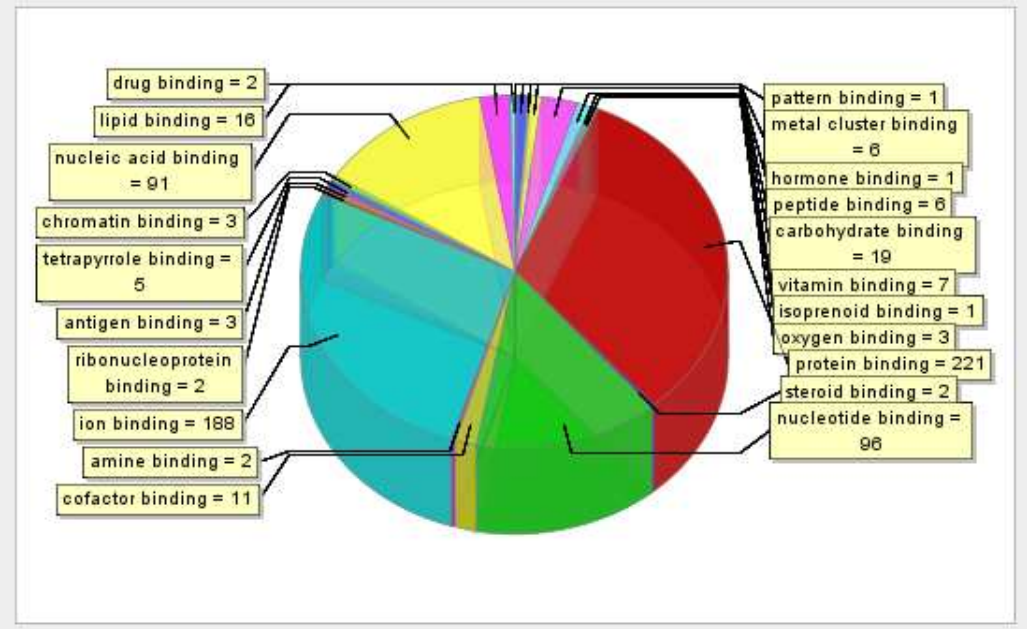


Figure 45 Breakdown of molecular binding

Catalytic activity was the other main category of processes that were shown to be differentially expressed in the dataset. Catalytic activity is further categorized in the figure 45.

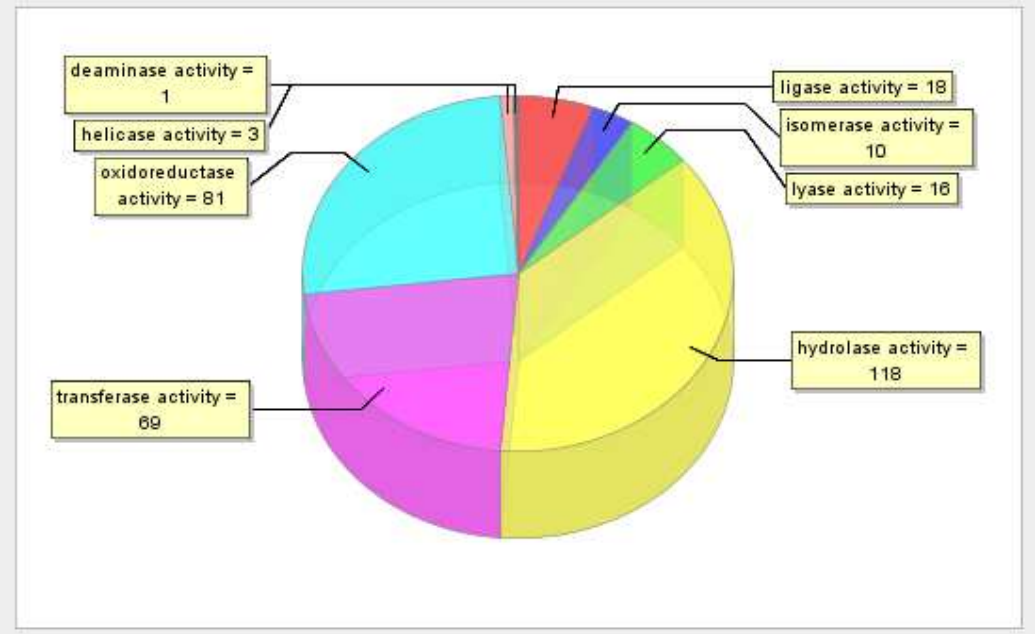


Figure 46 Catalytic processes

Hydrolase, oxidoreductase and transferase activity can be seen to contain the majority of differential expressed processes.

c) Biological Processes

Gene Ontology mapping using the SAM dataset (FDR of 5%) identified the following biological processes that were differentially expressed:

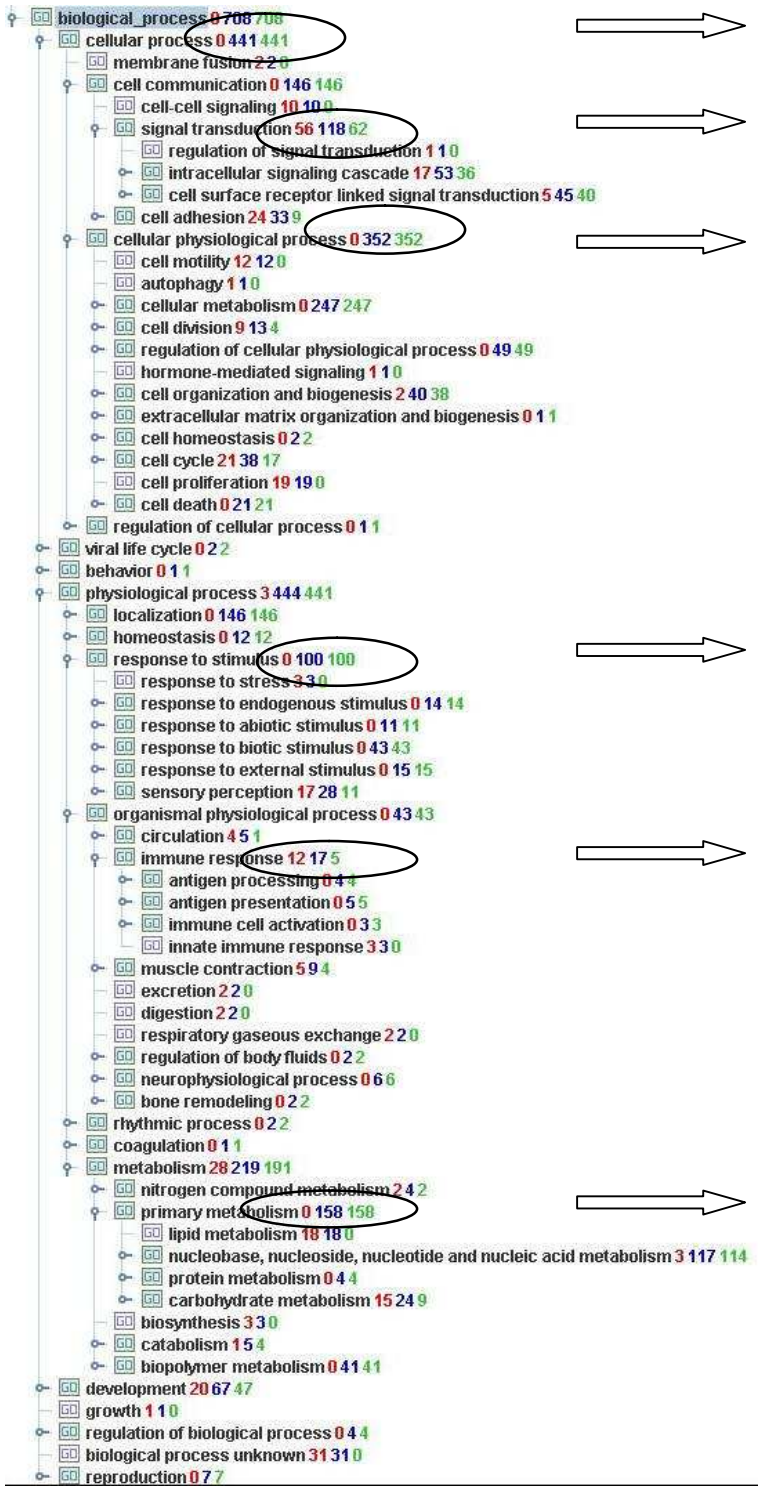


Figure 47 - GO-tree map of Biological Processes based on the deceased donor versus living donor (SAM FDR 5%) dataset.

In figure 46 the Go tree map for Biological processes is shown with its various components represented as a pie chart. Under biological processes and within the sub-term cellular processes, cell communication and cell physiological processes contained the greatest numbers of differentially expressed genes and are shown in figure 47.

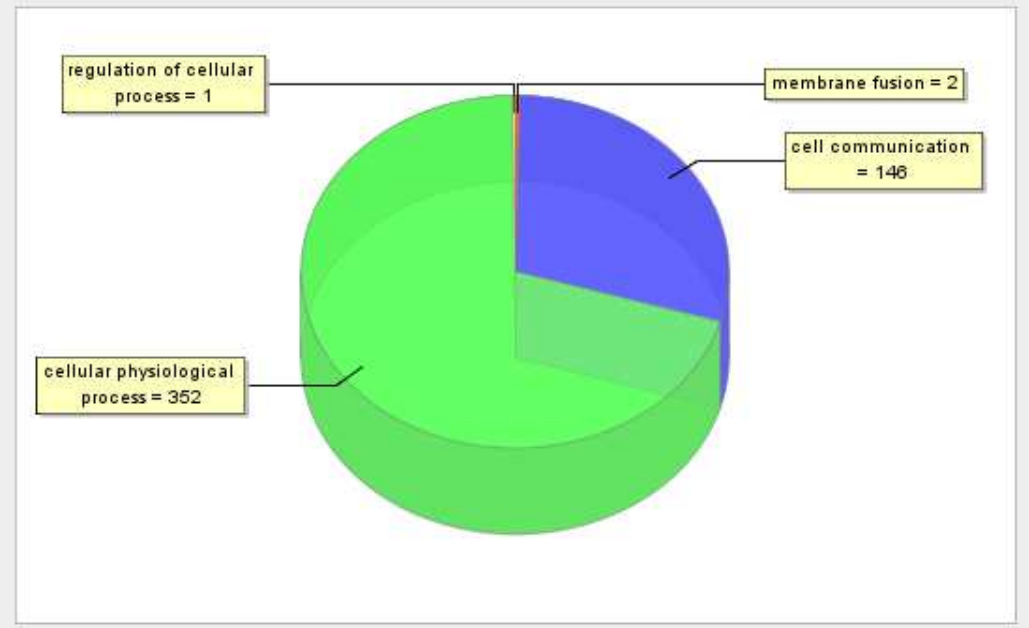


Figure 48 Cellular Processes – Breakdown

Within cell communication, signal transduction processes are further broken down and represented in figure 48 below.

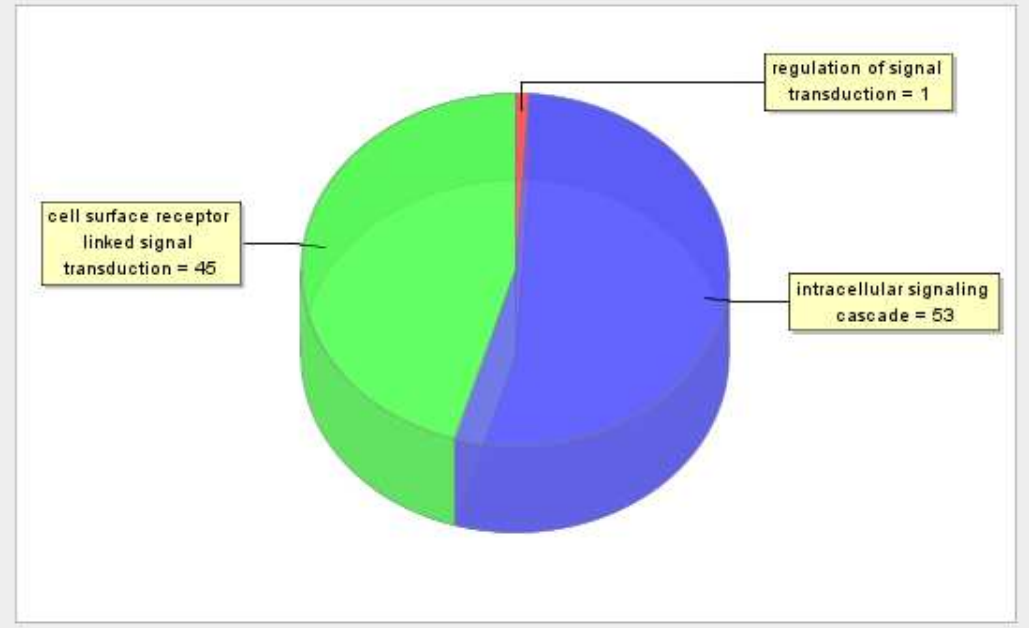


Figure 49 Signal Transduction

Figure 49 and 48 demonstrates that intracellular signalling and cell surface receptor linked signal transduction processes were the majority processes to be differentially expressed within cellular processes.

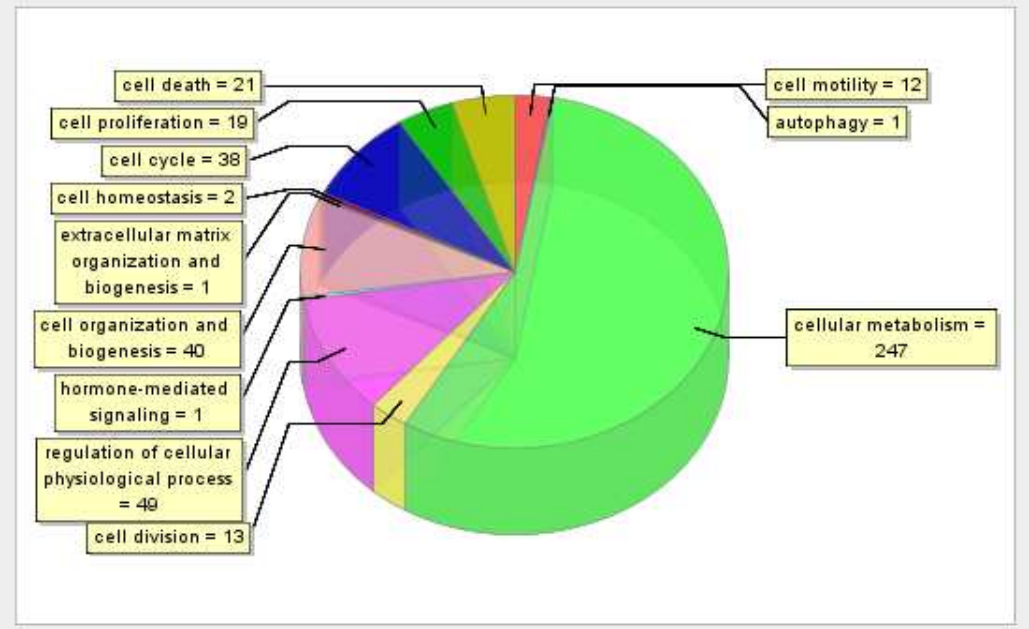


Figure 50 Cellular physiological processes

The second main group of differentially expressed processes under biological processes was cellular physiological processes. This group is broken down into its constituent components in figure49, cellular metabolism predominates.

The second main group of differentially expressed processes under biological processes was physiological processes. Under this term response to stimulus, immune response and (primary) metabolism terms contained the majority of differentially expressed genes and are shown in Figures 50, 51 and 52.

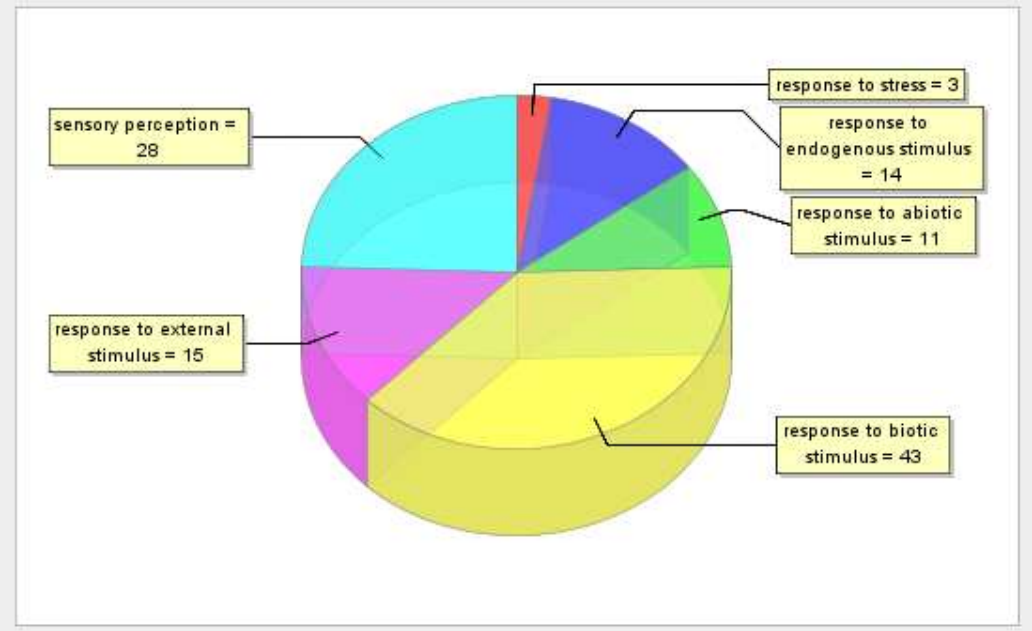


Figure 51 - Response to Stimulus

In figure 50 responses to biotic stimuli are seen to contain the largest number of differentially expressed genes.

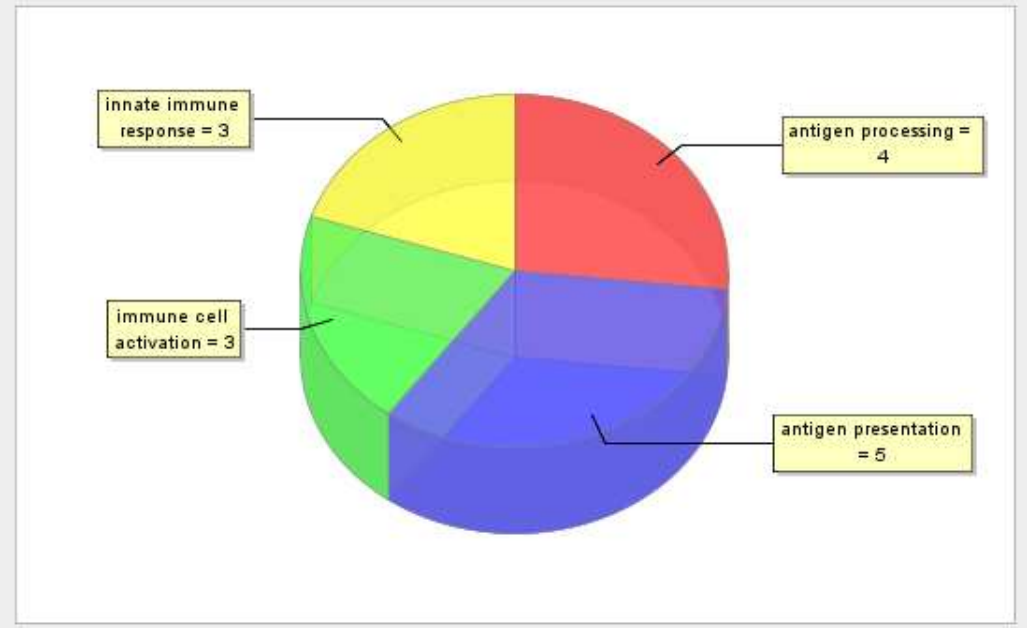


Figure 52 Immune responses

Differentially expressed genes were fairly equally distributed in the sub-terms antigen presentation, immune cell activation, innate immune response and antigen presentation in figure 51. A list of those genes is shown in table 16.

Accession	Description
NM_021983	HLA class II histocompatibility antigen, DR-W53 beta chain precursor.
NM_002121	HLA class II histocompatibility antigen, SB beta chain (Clone PHA- beta) (Fragment).
NM_000491	Complement C1q subcomponent, B chain precursor.
NM_021155	CD209 antigen (Dendritic cell-specific ICAM-3-grabbing nonintegrin 1)
NM_002120	HLA class II histocompatibility antigen, DO beta chain precursor (MHC class II antigen DOB).
NM_004951	EBV-induced G-protein coupled receptor 2 (EBI2).
NM_006084	Transcriptional regulator ISGF3 gamma subunit (IFN-alpha responsive transcription factor subunit).
NM_002818	Proteasome activator complex subunit 2 (Proteasome activator 28-beta subunit) (PA28beta).
NM_005849	immunoglobulin superfamily, member 6
NM_005776	Cornichon homolog (TGAM77) (UNQ155/PRO181).
NM_033554	HLA class II histocompatibility antigen, DP alpha chain precursor (HLA-SB alpha chain) (MHC class II DP3-alpha).
NM_005218	Beta-defensin 1 precursor (BD-1) (hBD-1) (Defensin, beta 1).
NM_025239	programmed cell death 1 ligand 2.
NM_001710	Complement factor B precursor (EC 3.4.21.47) (C3/C5 convertase) (Glycine-rich beta glycoprotein).
NM_015254	Kinesin-like protein KIF13B (Kinesin-like protein GAKIN).
NM_017413	Apelin precursor (APJ endogenous ligand).
NM_001300	Core promoter element-binding protein (Kruppel-like factor 6) (B-cell derived protein 1).
NM_001212	Complement component 1, Q subcomponent binding protein, mitochondrial precursor (Glycoprotein gC1qBP).

Table 16 - Significantly differentially expressed genes involved in the GO term Immune response

Under the sub-term metabolism, the majority processes that were differentially expressed were associated with nucleotide metabolism (figure 52).

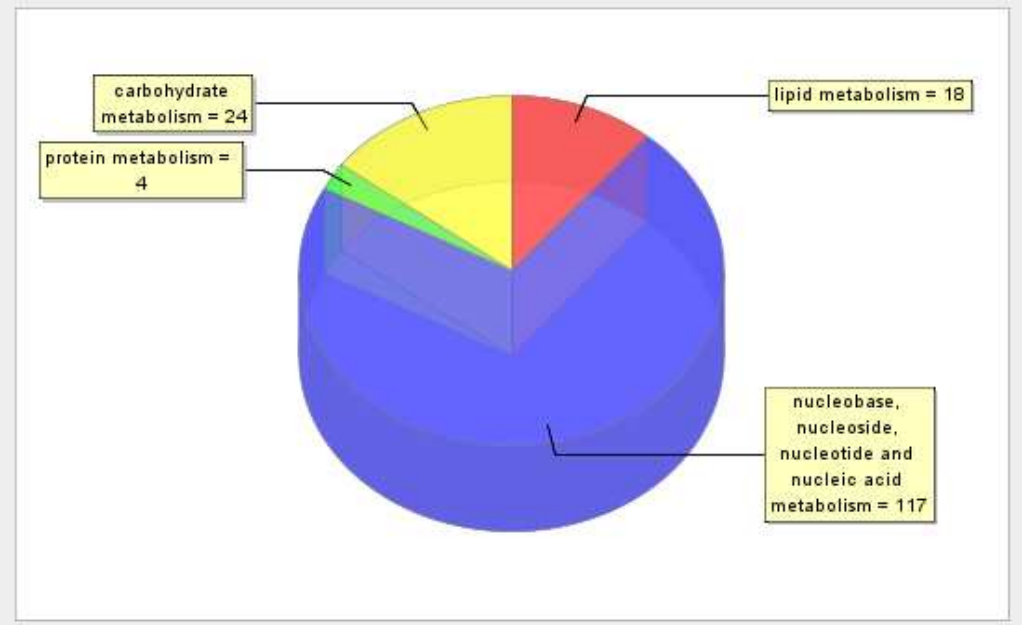


Figure 53 Primary metabolism

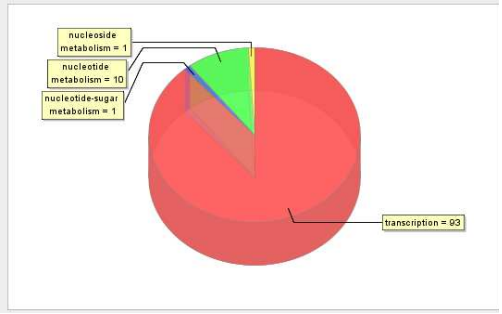


Figure 54 Breakdown of the chemical reactions involving nucleobases, nucleosides, nucleotides and nucleic acids

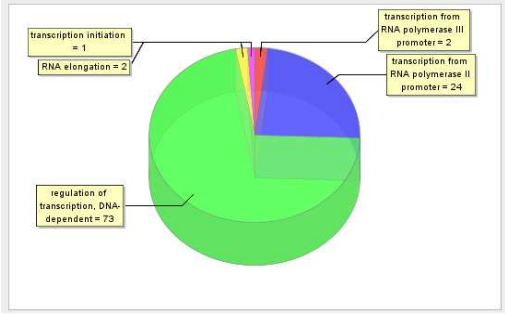


Figure 55 Breakdown of transcription, DNA-dependent

Processes involving nucleobases, nucleosides, nucleotides and nucleic acids are further broken down into their constituent components in Figure 53. Most of those transcription components were DNA dependant regulation transcription processes demonstrated in Figure 54.

3.4.1.4 Pathway analysis

Pathway Analysis was performed using Pathway Studio 5. The dataset identified using SAM analysis (which found significantly differentially expressed genes between the deceased and living donor groups, FDR 5%) was analysed in order to look for recognised interactions between the genes identified as being significantly differentially expressed. The overall gene interaction map is shown in figure 54.

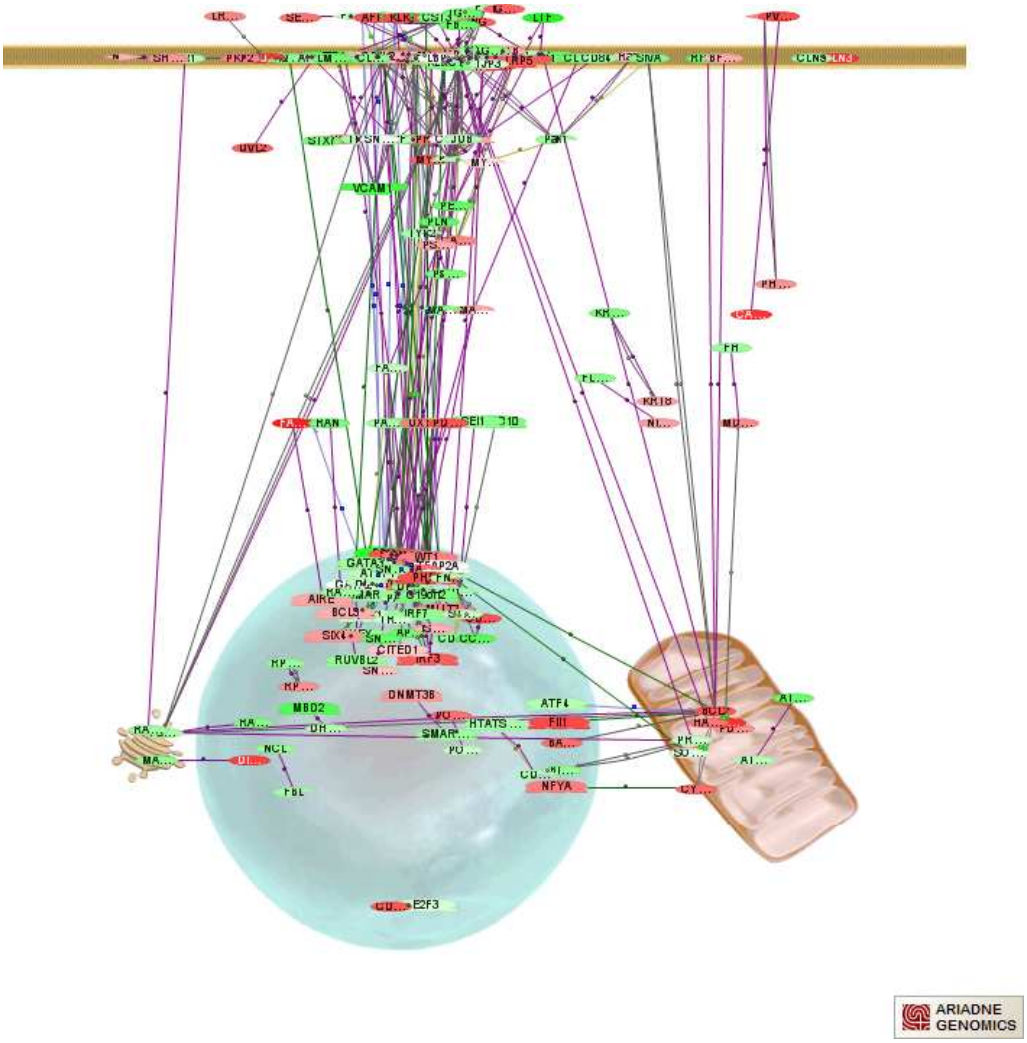


Figure 56 - Pathway created using all significantly differentially expressed genes identified by SAM (FDR 5%)

From this pathway numerous relations between genes were found that related to many cellular processes. An example is shown in table 17. This lists gene expression terms that were identified in the pathway shown in figure 56

Relation	Journal Link
FOS --+> ICAM1	http://atvb.ahajournals.org/cgi/content/full/19/9/2078
FOS --+> CCL2	http://atvb.ahajournals.org/cgi/content/full/19/9/2078
SMAD3 ---| CCL2	http://circres.ahajournals.org/cgi/content/full/94/5/601
SMAD3 ---> MYOG	http://embojournal.npgjournals.com/cgi/content/full
SMARCA4 ---| FOS	http://mcb.asm.org/cgi/content/full/19/4/2724
FOS ---> TIMP1	http://www.jbc.org/cgi/content/full/271/2/774
ELK1 --+> FOS	http://www.jbc.org/cgi/content/full/275/21/16064
FOS --+> EGF	http://www.jbc.org/cgi/content/full/276/48/45320
SMARCA4--+>CYP1A1	http://www.jbc.org/cgi/content/full/277/14/11821
TFAP2A ---> ICAM1	http://www.jbc.org/cgi/content/full/278/48/47498
SMAD4 ---> SMAD3	http://www.pnas.org/cgi/content/full/96/22/12442
ATF4 --+> BCL2	
CCL11 --+> VCAM1	
CCL11 ---> ICAM1	
CCL2 ---> ICAM1	
ATBF1 ---| AFP	

Table 17 - Gene expression terms identified by SAM (FDR 5%) in the pathway built using all significantly differentially expressed genes.

Interactions which have been previously described are shown as hyperlinks. Further pathway analysis was performed on the dataset produced by SAM (FDR = 5%). The following pathways were found to contain genes that were significantly differentially expressed in the dataset and of interest and relevance to kidney transplantation:

i. Death Receptor Pathway
ii. DR3 / DR4 Pathway
iii. Epidermal Growth Factor Signalling
iv. IFN-α / β / γ EGF
v. Inhibition of Apoptosis

i. Death Receptor Pathway

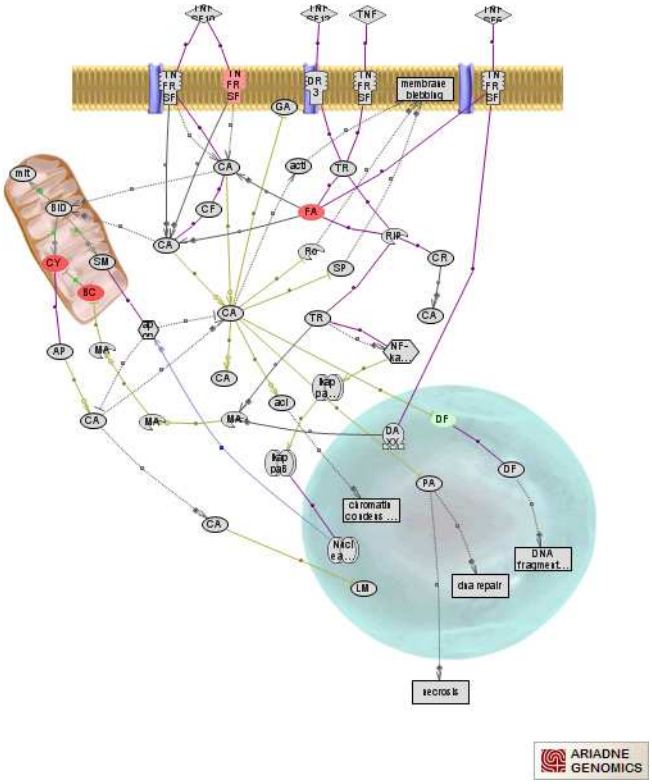


Figure 57 - Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Death Receptor Pathway. Red coloured genes are up-regulated and genes shown in green are down -regulated

Genes that were significantly differentially expressed in the dataset are shown in colour in the death receptor pathway (figure 57). A list of genes is given in table 18. The same genes were also found to be involved in the DR3 / DR4 Pathway.

Name	Description	LocusLink ID
BCL2	B-cell leukaemia/lymphoma 2	320761, 596, 320732
CYC1	cytochrome c-1 (predicted)	1537, 300047, 105730
DFFA	DNA fragmentation factor, alpha subunit	1676, 114214, 13347
FADD	Fas (TNFRSF6)-associated via death domain	14082, 114082, 8772, 266610,
TNFRSF10A	tumour necrosis factor receptor superfamily, member 10b (predicted)	8797, 21933, 364420

Table 18 - Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Death Receptor and DR3 / DR4 Pathway

ii. DR3 / DR4 Pathway

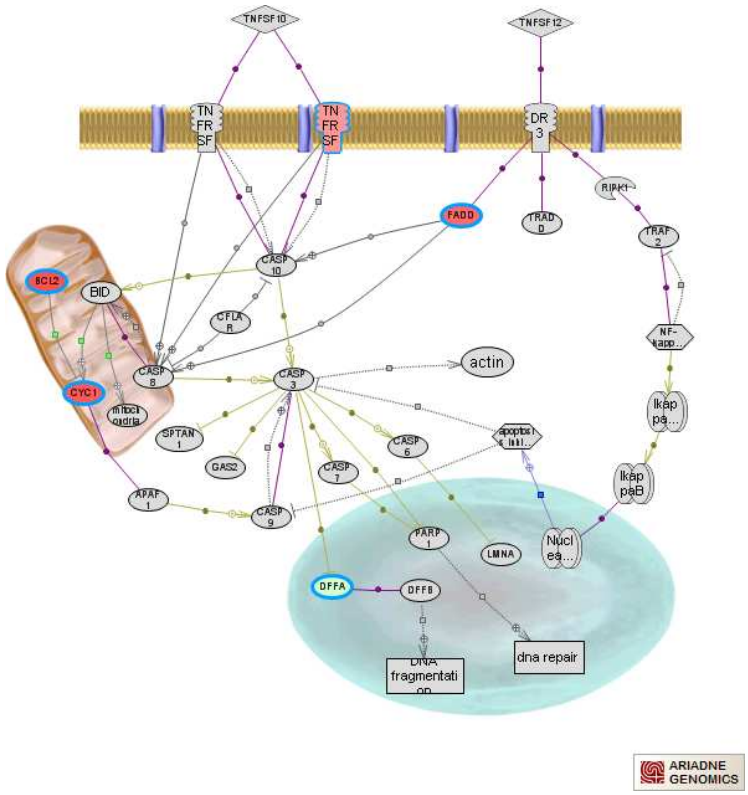


Figure 58 - Significantly differentially expressed genes identified by SAM (FDR 5%) found in the DR3 / DR4 Pathway. Red coloured genes are up-regulated and genes shown in green are down -regulated

The list of genes shown in the DR3/DR4 pathway is the same as those identified in the death receptor pathway and is given in table 18.

iii. Epidermal Growth Factor Signalling

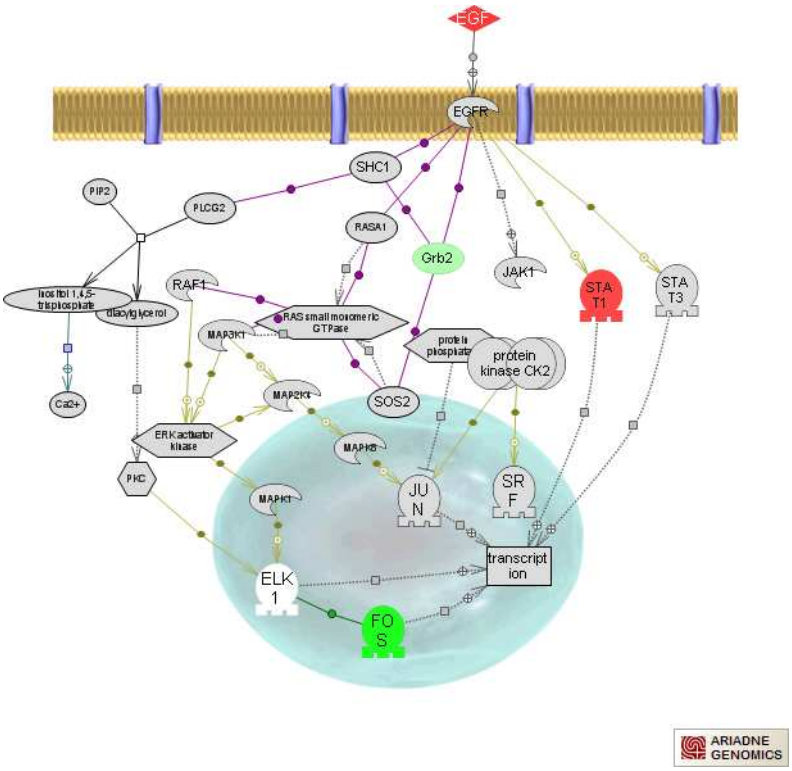


Figure 59 Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Epidermal Growth Factor Signalling. Red coloured genes are up-regulated and genes shown in green are down -regulated

Genes that were significantly differentially expressed in the dataset are shown in colour in the Epidermal Growth Factor Signalling (Figure 59). A list of genes is given in table 19.

Name	Description	LocusLink ID
ELK1	ELK1, member of ETS oncogene family	246325, 314436, 25170, 2002, 13712
FOS	FBJ murine osteosarcoma viral oncogene homolog	114281, 2353, 60585, 24371, 14281, 314322
STAT1	signal transducer and activator of transcription 1	68723, 20846, 98183, 6772, 445332, 320846, 25124, 99845
Grb2	growth factor receptor bound protein 2	81504, 414784, 308534, 103536, 80299,
EGF	epidermal growth factor	13645, 1950, 10976, 99717, 25313

Table 19 - Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Epidermal Growth Factor Signalling.

iv. Inhibition of Apoptosis

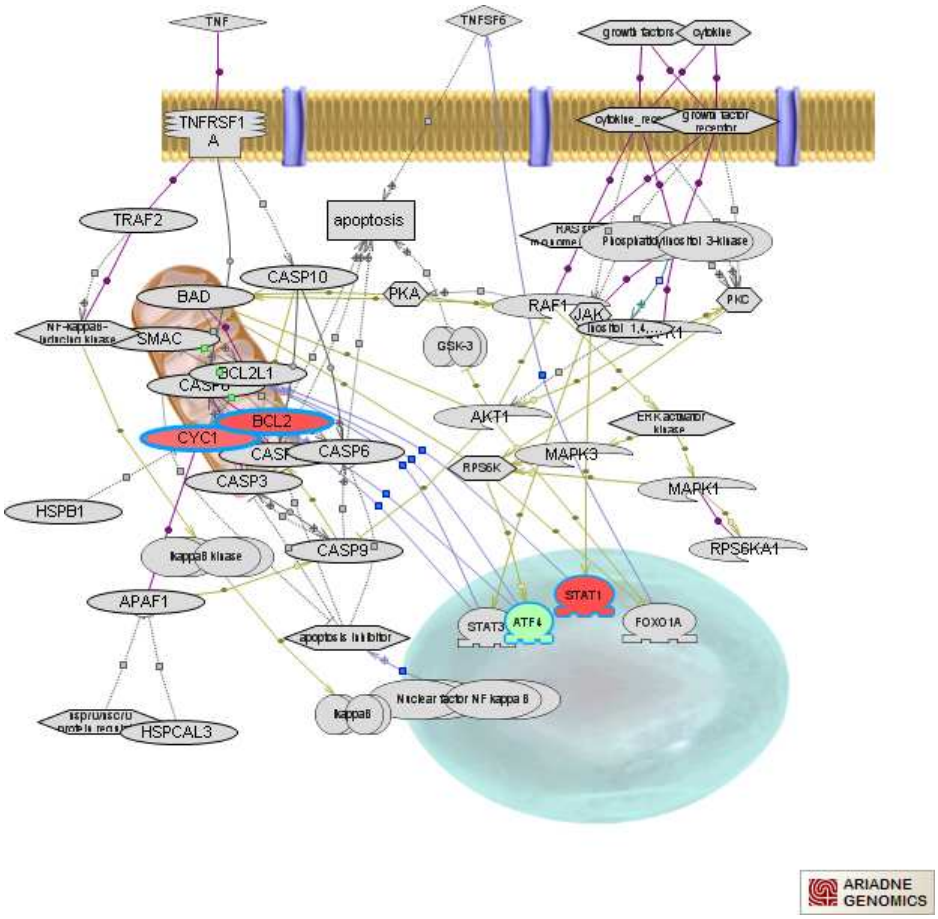


Figure 60 Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Inhibition of Apoptosis. Red coloured genes are up-regulated and genes shown in green are down -regulated

Genes that were significantly differentially expressed in the dataset are shown in colour in the Inhibition of Apoptosis pathway (Figure 60). A list of genes is given in table 20.

Name	Description	LocusLink ID
STAT1	signal transducer and activator of transcription 1	6772, 98183, 25124, 99845, 68723, 445332, 320846,
BCL2	B-cell leukaemia/lymphoma 2	320732, 596, 320761, 319577, 98734,
ATF4	activating transcription factor 4	79255, 468, 11911
CYC1	cytochrome c-1 (predicted)	1537, 300047, 105730, 66445

Table 20 - Significantly differentially expressed genes identified by SAM (FDR 5%) found in the Inhibition of Apoptosis pathway

v. IFN-α / β / γ EGF

Pathway analysis of the interferon α, β, γ and epidermal growth factor pathways identified STAT1 (signal transducer and activator of transcription 1) as a common gene differentially expressed in the pathways. The protein encoded by this gene is a member of the STAT protein family.

Expanded analysis of all significantly differentially expressed genes

The common genes (1802) were entered back into the pathway analysis algorithm. In order to examine the wider interactions of identified genes terms were selected to expand the pathway to include other genes that would potentially fill in or bridge gaps between pathways connected to each other. These pathways were likely to only contain a few significantly differentially expressed genes were in the dataset because the other genes had been removed in the filtering and normalisation processes described earlier.

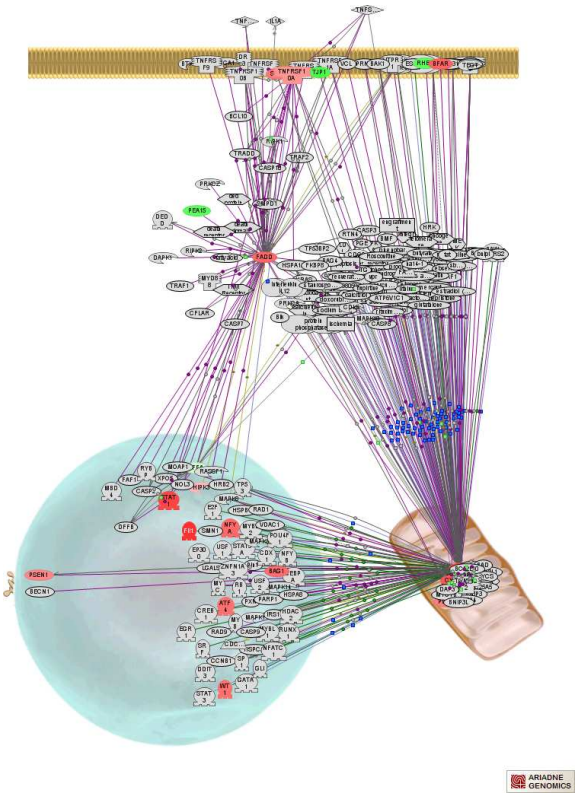


Figure 61- Expanded Pathway

Figure 61 shows an expanded pathway based upon the differentially expressed genes found in the dataset. Genes found in the dataset are coloured either red or green depending on them being up or down-regulated. Coloured genes are those genes in the dataset. Genes in grey are the genes that fill in the gaps in the pathway.

Name	Description	LocusLink ID
STAT1	signal transducer and activator of transcription 1	20846, 98183, 6772, 25124, 99845, 68723, 445332, 320846
BCL2	B-cell leukaemia/lymphoma 2	319577, 596, 320732, 98734
ATF4	activating transcription factor 4	11911, 79255, 468
Fli1	Friend leukaemia integration 1	2313, 315532, 14247
WT1	Wilms tumour 1	294807, 213439, 7490, 319408,
TNFRSF10A	tumour necrosis factor receptor superfamily, member 10b (predicted)	21933, 364420, 8797
PSEN1	presenilin 1	29192, 19164, 5663
HIPK3	homeodomain interacting protein kinase 3	83617, 10114, 15259
PDCD8	programmed cell death 8	83533, 26926, 15459, 9131
CYC1	cytochrome c-1 (predicted)	1537, 300047, 105730, 66445
FADD	Fas (TNFRSF6)-associated via death domain	266610, 14082, 8772, 414082, 114082
PEA15	phosphoprotein enriched in astrocytes 15	364052, 17177, 18611, 29891, 8682
BAG1	Bcl2-associated athanogene 1 (predicted)	12017, 297994, 573
TJP1	tight junction protein 1 (predicted)	101244, 7082, 107526, 21872,
DFFA	DNA fragmentation factor, alpha subunit	13347, 1676, 114214
SIVA	Cd27 binding protein (predicted)	362791, 30954, 10572, 220525
RHEB	RAS-homolog enriched in brain	26954, 6009, 19744
NFYA	nuclear transcription factor-Y alpha	4800, 106471, 18044, 29508
BFAR	bifunctional apoptosis regulator	67118, 304709, 106253, 106390,

Table 21 - Expanded Pathway

Table 21 lists those differentially expressed genes present in the expanded pathway shown in figure 60 that were in the dataset.

Pathway analysis of dataset comparing those up-regulated versus down-regulated genes from the SAM dataset following Hierarchical clustering (see figure 37).

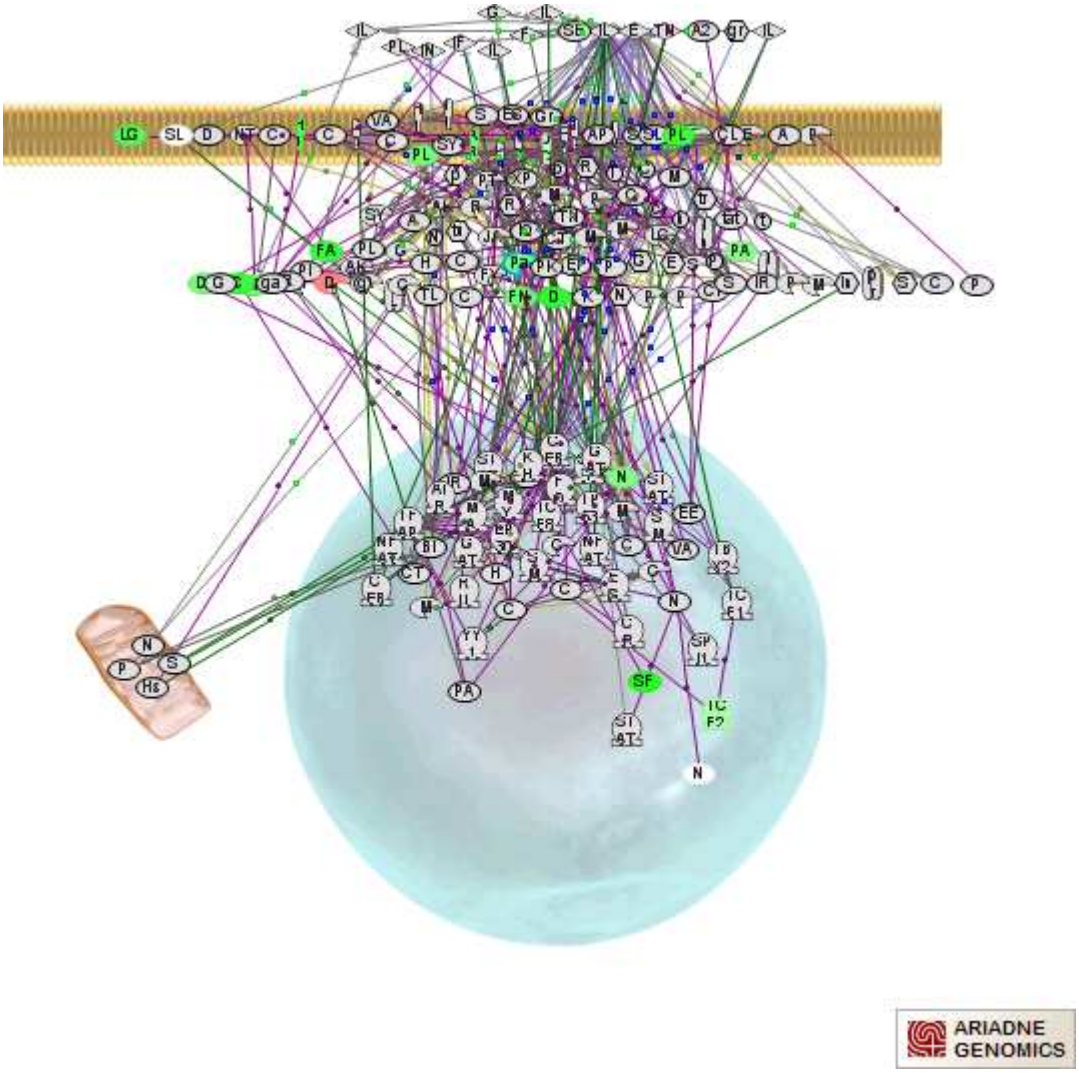


Figure 62 Pathway created from up vs. down-regulated genes following hierarchical clustering

Figure 62 is the pathway created from the dataset (FDR 0%) shown in Figure 38. Hierarchical clustering revealed two groups of genes. Cluster A represented genes that were down-regulated in the deceased donor group compared to the living group. Cluster B represents genes that were up-regulated in the deceased versus living donor group. Most of the genes shown in Figure 62 are from cluster A and are shown in green.

In Figure 63 both clusters of genes are compared to the literature using Medscan and the search terms 'transplantation' and 'inflammation'. The figure shows the interactions between genes identified and there cellular interactions described in the literature.

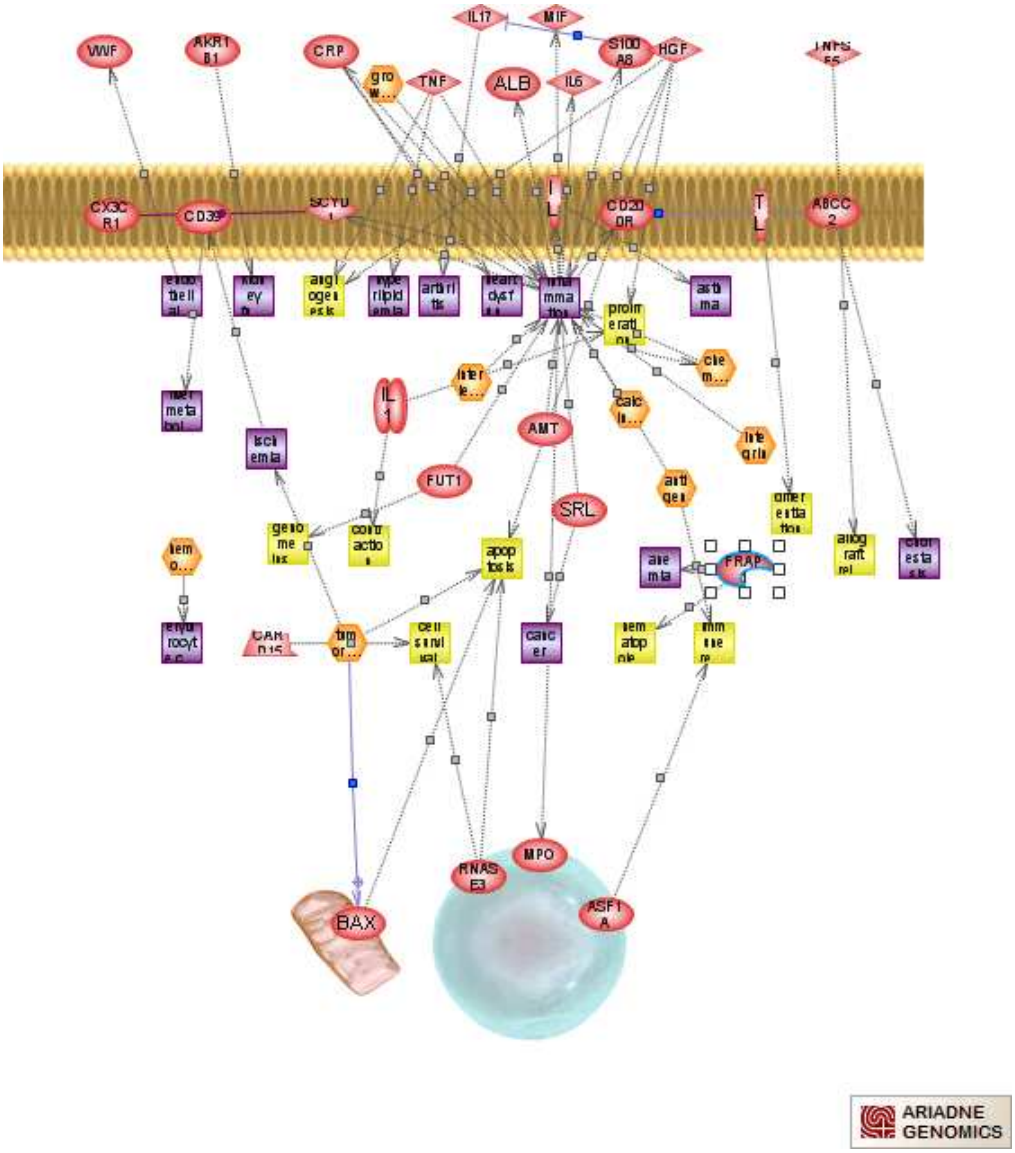


Figure 63 – Relations of identified genes to the search terms inflammation and transplantation.

3.4.2 Deceased Donors (Immediate function) versus all donors (Deceased and Living) with Moderate / Poor / Non-function

The purpose of this sub-analysis was to potentially identify genes potentially involved in processes that were inherent to the graft or occurred at the time of biopsy prior to the process becoming apparent clinically.

3.4.2.1 Significance Analysis of Microarrays

SAM analysis was performed and identified 109 significantly differentially expressed genes .These are shown in table 22; unidentified and hypothetical genes are excluded.

ID	Description
NM_001010	40S ribosomal protein S6 (Phosphoprotein NP33). [Source:Uniprot/SWISSPROT;Acc:P62753]
NM_000991	60S ribosomal protein L28. [Source:Uniprot/SWISSPROT;Acc:P46779]
NM_000998	60S ribosomal protein L37a. [Source:Uniprot/SWISSPROT;Acc:P61513]
NM_001885	Alpha crystallin B chain (Alpha (B)-crystallin) (Rosenthal fiber component) (Heat-shock protein beta-5) (HspB5). [Source:Uniprot/SWISSPROT;Acc:P02511]
NM_002372	Alpha-mannosidase II (EC 3.2.1.114) (Mannosyl-oligosaccharide 1,3-1,6-alpha-mannosidase) (MAN II) (Golgi alpha-mannosidase II) (Mannosidase alpha class 2A member 1). [Source:Uniprot/SWISSPROT;Acc:Q16706]
NM_178510	ankyrin repeat and kinase domain containing 1 [Source:RefSeq_peptide;Acc:NP_848605]
NM_017413	Apelin precursor (APJ endogenous ligand). [Source:Uniprot/SWISSPROT;Acc:Q9ULZ1]
NM_001686	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14). [Source:Uniprot/SWISSPROT;Acc:P06576]
NM_006476	ATP synthase g chain, mitochondrial (EC 3.6.3.14) (ATPase subunit G). [Source:Uniprot/SWISSPROT;Acc:O75964]
NM_001697	ATP synthase oligomycin sensitivity conferral protein, mitochondrial precursor (EC 3.6.3.14) (OSCP). [Source:Uniprot/SWISSPROT;Acc:P48047]
NM_005178	B-cell lymphoma 3-encoded protein (Bcl-3 protein). [Source:Uniprot/SWISSPROT;Acc:P20749]
NM_021211	Buster1 transposase-like protein [Source:RefSeq_peptide;Acc:NP_067034]

NM_016395	butyrate-induced transcript 1 [Source:RefSeq_peptide;Acc:NP_057479]
NM_005956	C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase) [Includes: Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5)
NM_001780	CD63 antigen (Melanoma-associated antigen ME491) (Lysosome-associated membrane glycoprotein 3) (LAMP-3) (Ocular melanoma-associated antigen) (OMA81H) (Granulophysin). [Source:Uniprot/SWISSPROT;Acc:P08962]
NM_018491	COBW domain containing 1 [Source:RefSeq_peptide;Acc:NP_060961]
NM_001866	Cytochrome c oxidase polypeptide VIIb, mitochondrial precursor (EC 1.9.3.1). [Source:Uniprot/SWISSPROT;Acc:P24311]
NM_006360	dendritic cell protein [Source:RefSeq_peptide;Acc:NP_006351]
NM_022365	DnaJ homolog subfamily C member 1. [Source:Uniprot/SWISSPROT;Acc:Q96KC8]
NM_031208	fumarylacetoacetate hydrolase domain containing 1 [Source:RefSeq_peptide;Acc:NP_112485]
NM_015710	Glioma tumour suppressor candidate region gene 2 protein (p60). [Source:Uniprot/SWISSPROT;Acc:Q9NZM5]
NM_014367	growth and transformation-dependent protein (E2IG5), mRNA [Source:RefSeq_dna;Acc:NM_014367]
NM_016292	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumour necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1). [Source:Uniprot/SWISSPROT;Acc:Q12931]
NM_004499	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B) (APOBEC-1 binding protein 1) (ABBP-1). [Source:Uniprot/SWISSPROT;Acc:Q99729]
NM_001859	High-affinity copper uptake protein 1 (hCTR1) (Copper transporter 1) (Solute carrier family 31, member 1). [Source:Uniprot/SWISSPROT;Acc:O15431]
NM_018951	Homeobox protein Hox-A10 (Hox-1H) (Hox-1.8) (PL). [Source:Uniprot/SWISSPROT;Acc:P31260]
NM_006774	Indolethylamine N-methyltransferase (EC 2.1.1.49) (Aromatic alkylamine N-methyltransferase) (Indolamine N-methyltransferase) (Arylamine N-methyltransferase) (Amine N-methyltransferase). [Source:Uniprot/SWISSPROT;Acc:O95050]
NM_080730	intermediate filament-like protein MGC:2625 isoform 3 [Source:RefSeq_peptide;Acc:NP_542769]
NM_031959	Keratin-associated protein 3-2 (Keratin-associated protein 3.2) (High sulfur keratin-associated protein 3.2). [Source:Uniprot/SWISSPROT;Acc:Q9BYR7]
NM_018269	membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 [Source:RefSeq_peptide;Acc:NP_060739]

NM_020191	Mitochondrial 28S ribosomal protein S22 (S22mt) (MRP-S22) (GK002). [Source:Uniprot/SWISSPROT;Acc:P82650]
NM_004279	Mitochondrial processing peptidase beta subunit, mitochondrial precursor (EC 3.4.24.64) (Beta-MPP) (P-52). [Source:Uniprot/SWISSPROT;Acc:O75439]
NM_023948	motile sperm domain containing 3 [Source:RefSeq_peptide;Acc:NP_076438]
NM_025107	myc target 1 [Source:RefSeq_peptide;Acc:NP_079383]
NM_004551	NADH-ubiquinone oxidoreductase 30 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-30KD) (CI-30KD). [Source:Uniprot/SWISSPROT;Acc:O75489]
NM_004546	NADH-ubiquinone oxidoreductase AGGG subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-AGGG) (CI-AGGG). [Source:Uniprot/SWISSPROT;Acc:O95178]
NM_002490	NADH-ubiquinone oxidoreductase B14 subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-B14) (CI-B14). [Source:Uniprot/SWISSPROT;Acc:P56556]
NM_002489	NADH-ubiquinone oxidoreductase MLRQ subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-MLRQ) (CI-MLRQ). [Source:Uniprot/SWISSPROT;Acc:O00483]
NM_024824	nuclear protein UKp68 isoform 3 [Source:RefSeq_peptide;Acc:NP_997544]
NM_003611	Oral-facial-digital syndrome 1 protein (Protein 71-7A). [Source:Uniprot/SWISSPROT;Acc:O75665]
NM_000582	Osteopontin precursor (Bone sialoprotein 1) (Secreted phosphoprotein 1) (SPP-1) (Urinary stone protein) (Nephropontin) (Uropontin). [Source:Uniprot/SWISSPROT;Acc:P10451]
NM_006406	Peroxiredoxin 4 (EC 1.11.1.-) (Prx-IV) (Thioredoxin peroxidase AO372) (Thioredoxin-dependent peroxide reductase A0372) (Antioxidant enzyme AOE372) (AOE37-2). [Source:Uniprot/SWISSPROT;Acc:Q13162]
NM_004905	Peroxiredoxin 6 (EC 1.11.1.-) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2)
NM_020422	promethin [Source:RefSeq_peptide;Acc:NP_065155]
NM_000954	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin D synthase) (Glutathione-independent PGD synthetase)
NM_002792	Proteasome subunit alpha type 7 (EC 3.4.25.1) (Proteasome subunit RC6- 1) (Proteasome subunit XAPC7). [Source:Uniprot/SWISSPROT;Acc:O14818]
NM_006849	Protein disulfide-isomerase A2 precursor (EC 5.3.4.1) (PDIp). [Source:Uniprot/SWISSPROT;Acc:Q13087]
NM_003950	Proteinase activated receptor 4 precursor (PAR-4) (Thrombin receptor- like 3) (Coagulation factor II receptor-like 3). [Source:Uniprot/SWISSPROT;Acc:Q96RI0]

NM_020975	Proto-oncogene tyrosine-protein kinase receptor ret precursor (EC 2.7.1.112) (C-ret). [Source:Uniprot/SWISSPROT;Acc:P07949]
NM_025234	recombination protein REC14 [Source:RefSeq_peptide;Acc:NP_079510]
NM_015952	RWD domain containing protein 1 (CGI-24) (PTD013). [Source:Uniprot/SWISSPROT;Acc:Q9H446]
NM_021947	Serine racemase (EC 5.1.1.-). [Source:Uniprot/SWISSPROT;Acc:Q9GZT4]
NM_013376	SERTA domain-containing protein 1 (Transcriptional regulator interacting with the PHD-bromodomain 1) (TRIP-Br1) (CDK4-binding protein p34SEI1) (SEI-1). [Source:Uniprot/SWISSPROT;Acc:Q9UHV2]
NM_004175	Small nuclear ribonucleoprotein Sm D3 (snRNP core protein D3) (Sm-D3). [Source:Uniprot/SWISSPROT;Acc:P62318]
NM_005827	solute carrier family 35, member B1 [Source:RefSeq_peptide;Acc:NP_005818]
NM_014426	Sorting nexin 5. [Source:Uniprot/SWISSPROT;Acc:Q9Y5X3]
NM_017455	stromal cell derived factor receptor 1 isoform a [Source:RefSeq_peptide;Acc:NP_059429]
NM_003849	Succinyl-CoA ligase [GDP-forming] alpha-chain, mitochondrial precursor (EC 6.2.1.4) (Succinyl-CoA synthetase, alpha chain) (SCS-alpha). [Source:Uniprot/SWISSPROT;Acc:P53597]
NM_004711	Synaptogyrin-1. [Source:Uniprot/SWISSPROT;Acc:O43759]
NM_006430	T-complex protein 1, delta subunit (TCP-1-delta) (CCT-delta) (Stimulator of TAR RNA binding). [Source:Uniprot/SWISSPROT;Acc:P50991]
NM_014138	transmembrane protein 29 [Source:RefSeq_peptide;Acc:NP_054857]
NM_013262	Ubiquitin ligase MYLIP (EC 6.3.2.-) (Myosin regulatory light chain interacting protein) (MIR) (BM023). [Source:Uniprot/SWISSPROT;Acc:Q8WY64]
NM_014709	ubiquitin specific protease 34 [Source:RefSeq_peptide;Acc:NP_055524]
NM_003945	Vacuolar ATP synthase subunit H (EC 3.6.3.14) (V-ATPase H subunit) (Vacuolar proton pump H subunit) (V-ATPase M9.2 subunit) (V-ATPase 9.2 kDa membrane accessory protein). [Source:Uniprot/SWISSPROT;Acc:O15342]
NM_001183	Vacuolar ATP synthase subunit S1 precursor (EC 3.6.3.14) (V-ATPase S1 subunit) (V-ATPase S1 accessory protein) (V-ATPase Ac45 subunit) (XAP-3). [Source:Uniprot/SWISSPROT;Acc:Q15904]

Table 22 - Significantly differentially expressed genes identified by SAM (FDR 5%) in the deceased donor immediate function vs. non-immediately functioning kidneys

3.4.2.2 Hierarchical clustering

Hierarchical clustering of genes identified in table 22 is shown in Figure 64.

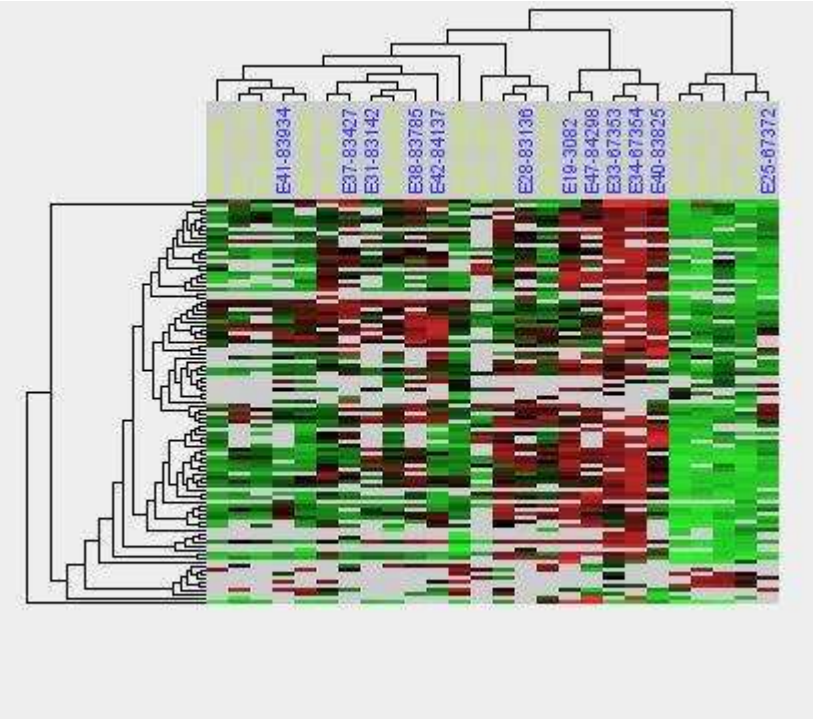


Figure 64 - Hierarchical clustering of significantly differentially expressed genes identified by SAM. Green column labels represent deceased donors (immediate function) and blue column labels represent all donors (non-immediate function)

Hierarchical clustering did not reveal any distinct groups or clusters within genes or samples. The data was therefore analysed using gene ontology mapping to see how the identified genes were distributed in terms of cellular function.

3.4.2.3 Gene Ontology

The following Gene Ontology components were identified from the dataset and are mapped in Figure 65.

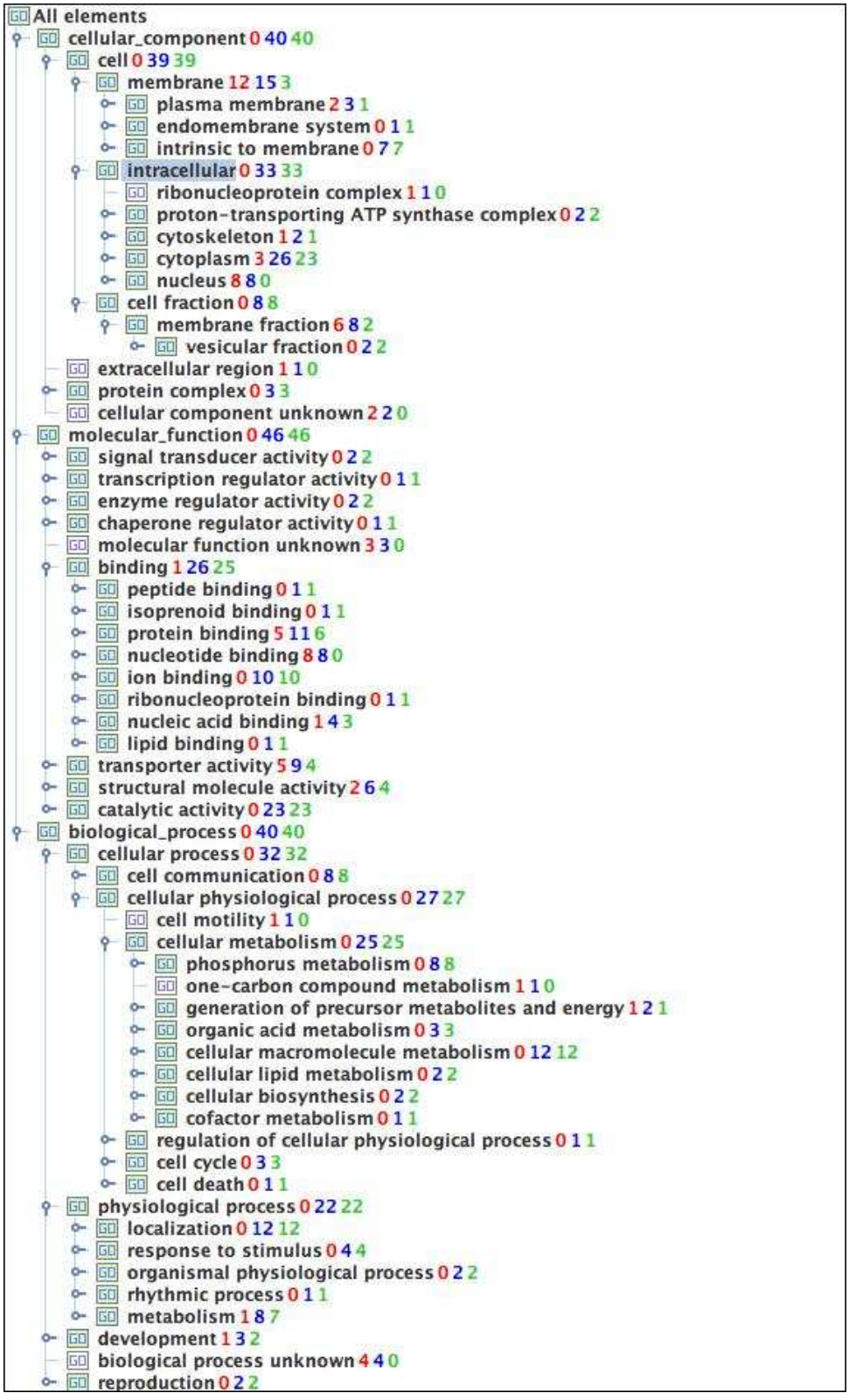


Figure 65 – The figure shows a GO tree map of the dataset Deceased donors (immediate function) vs. Non-immediately functioning donor kidneys (Moderate / Poor / Non-function)

The following intracellular processes and molecular functions were significantly differentially expressed and are shown in Figure 66 and Figure 67.

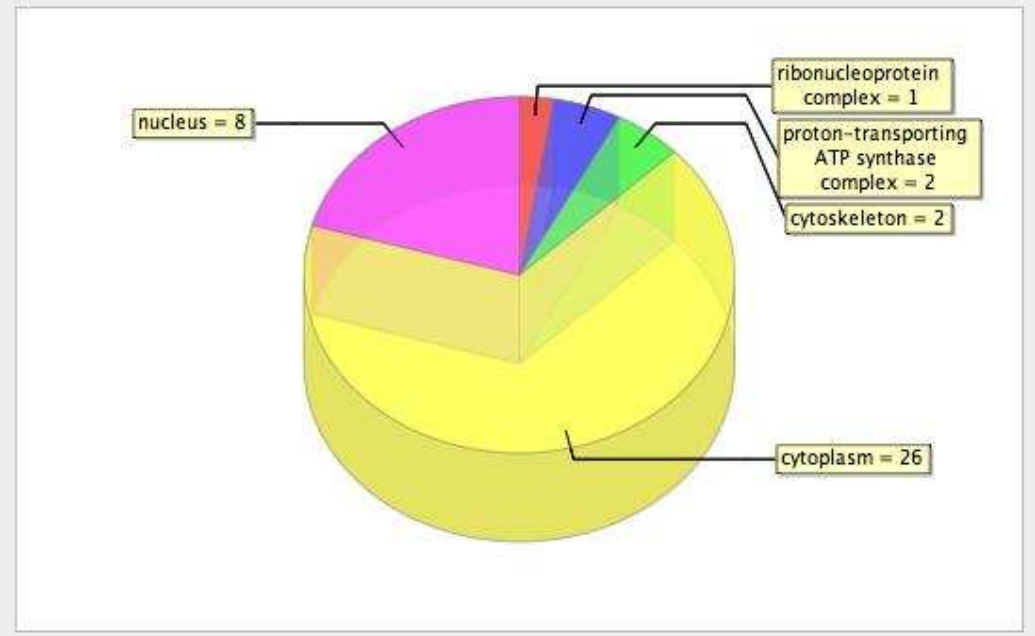


Figure 66 Pie Chart representing cellular processes (intracellular)

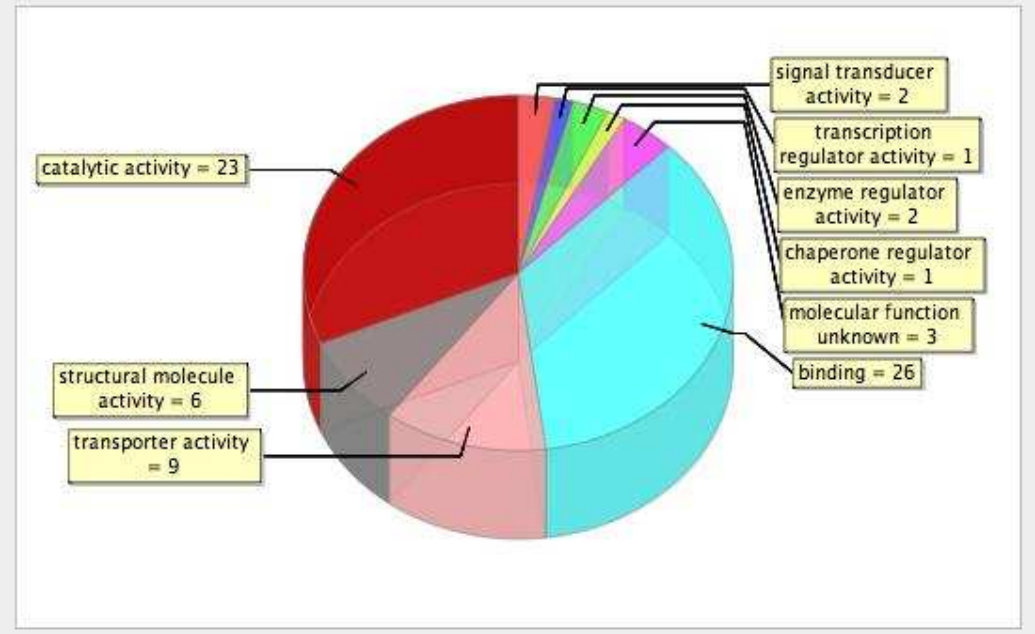


Figure 67 - Molecular function

Within the dataset Deceased donors (immediate function) vs. Non-immediately functioning donor kidneys (Moderate / Poor / Non-function) the following biological and cellular physiological processes were significantly differentially expressed and are shown in Figure 68 and Figure 69.

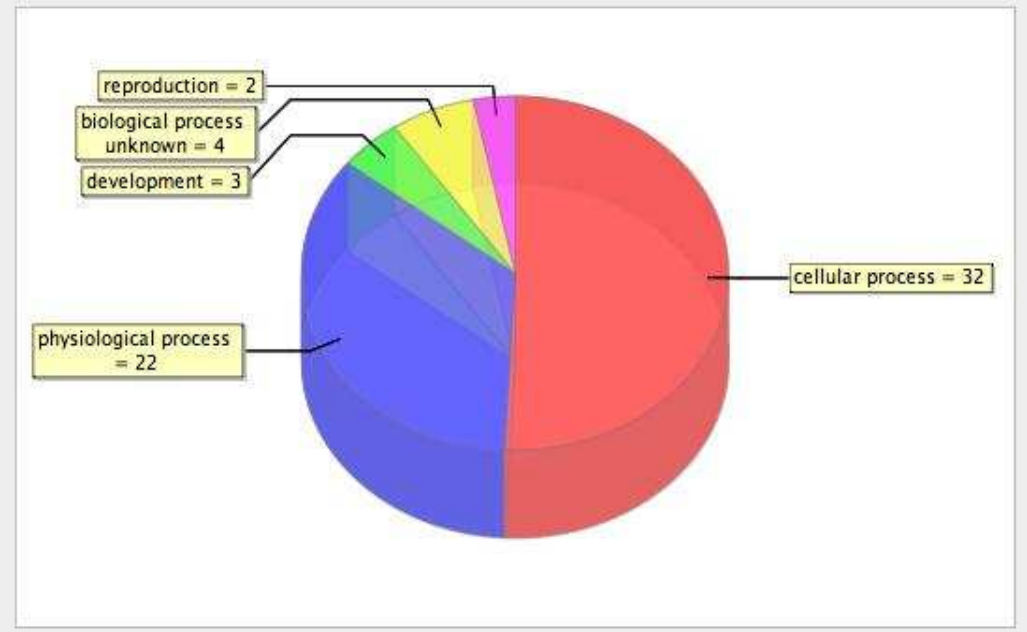


Figure 68 - Biological processes

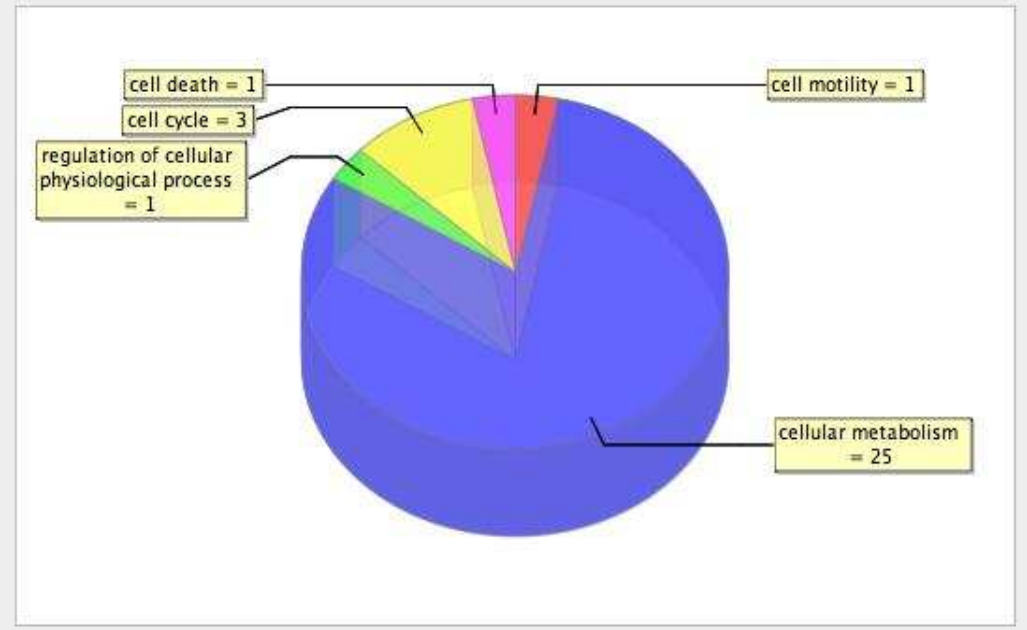


Figure 69 - Cellular physiological processes

In Figure 65 it can be seen that there are a number of unknown genes or genes that have not been ascribed a function. These are shown in table 23.

GENE ID	DESCRIPTION (CELLULAR PROCESSES UNKNOWN)
NM_016292	Tumour necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR- associated protein 1).
NM_003611	Oral-facial-digital syndrome 1 protein (Protein 71-7A).
GENE ID	DESCRIPTION (BIOLOGICAL PROCESSES UNKNOWN)
NM_016292	(Tumour necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR- associated protein 1).
NM_003611	Oral-facial-digital syndrome 1 protein (Protein 71-7A).
NM_015710	Glioma tumor suppressor candidate region gene 2 protein (p60).
NM_020191	Mitochondrial 28S ribosomal protein S22 (S22mt) (MRP-S22) (GK002).

Table 23 - Genes found to be significantly differentially expressed in the cadaveric donor immediate function vs. all non-immediately functioning kidneys (SAM FDR 5%) dataset classified as no cellular or biological function by Gene ontology

3.4.2.4 Pathway analysis

The 109 significantly differentially expressed genes were analysed further in pathway studio 5. The following significantly differentially expressed genes that interacted directly with one another are shown in table 24.

Name	Description	LocusLink ID
RET	ret proto-oncogene	5979, 24716, 19713
SPP1	secreted phosphoprotein 1	25353, 100557, 20750, 100728
BCL3	similar to B-cell leukaemia /lymphoma 3	101615, 12051, 680611, 112051, 101481, 292700, 602
PTGDS	prostaglandin D2 synthase	25526, 5730, 19215
CRYAB	crystallin, alpha B	25420, 1410, 12955
HOXA10	Homeobox protein A10	312338, 221882, 15395, 3206
PRDX6	peroxiredoxin 6	9588, 94167, 23804, 320807
CD63	CD63 antigen	967, 98116, 98099, 29186, 12512
DNAJC1	DnaJ (Hsp40) homolog, subfamily C, member 1	70841, 291369, 319395, 13418, 98831, 64215

Table 24 - Direct interactions between significantly differentially expressed genes in the Deceased donor (immediate function) vs. Non-immediately functioning donor kidneys (Moderate / Poor / Non-function) .

The genes interactions shown in table 24 are depicted in Figure 70.

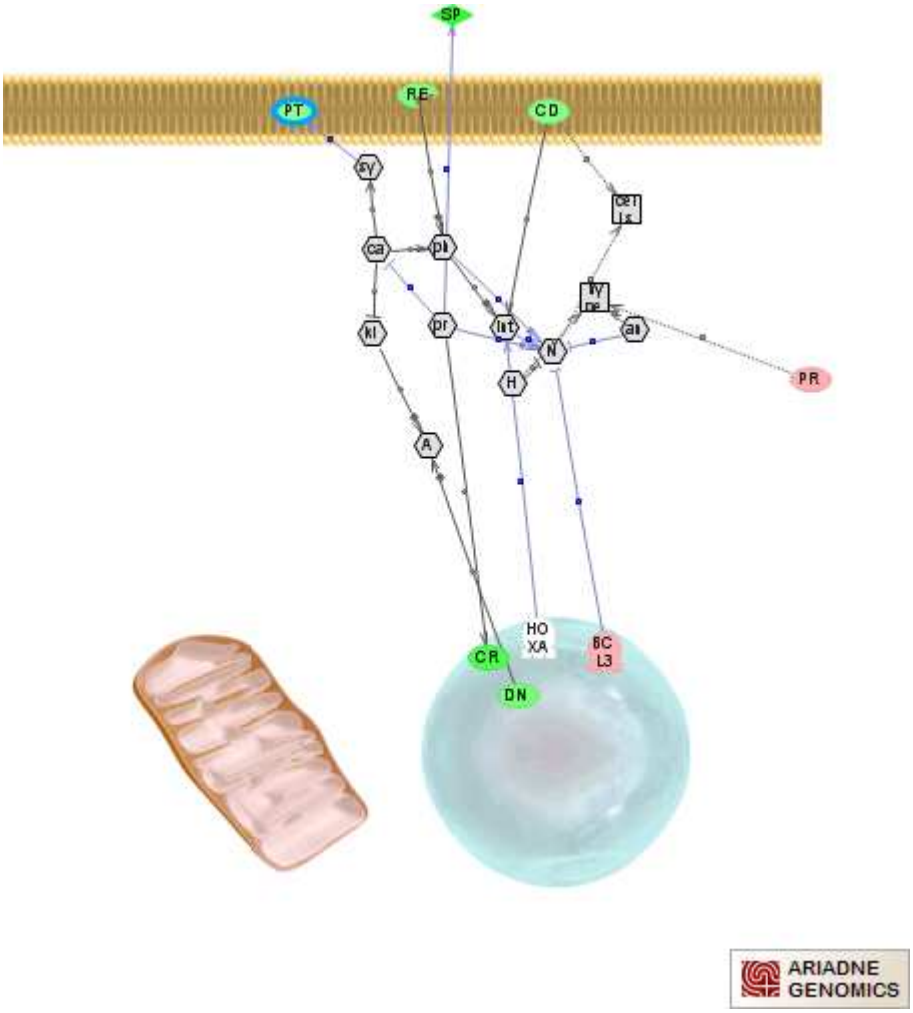


Figure 70 – Pathway analysis view of directly interacting genes found in the Deceased donor (immediate function) vs. Non-immediately functioning donor kidneys (Moderate / Poor / Non-function) dataset.

3.4.3 Deceased Donors (Immediate function) vs. Deceased donors: moderate poor /non function.

The purpose of this sub-analysis was to look for genes that were significantly differentially expressed that would indicate processes that were up or down-regulated in either group. An upregulated process in the deceased donor group with immediate function would be deemed "protective". A downregulated process in this group may be thought to have on otherwise damaging role. Conversely an upregulated process in the deceased group with poor or non-function would be deemed as "damaging" whereas a downregulated process may have a protective role.

3.4.3.1 Significance Analysis of Microarrays

Only eight genes were identified with a 0% FDR and a represented in Figure 71.

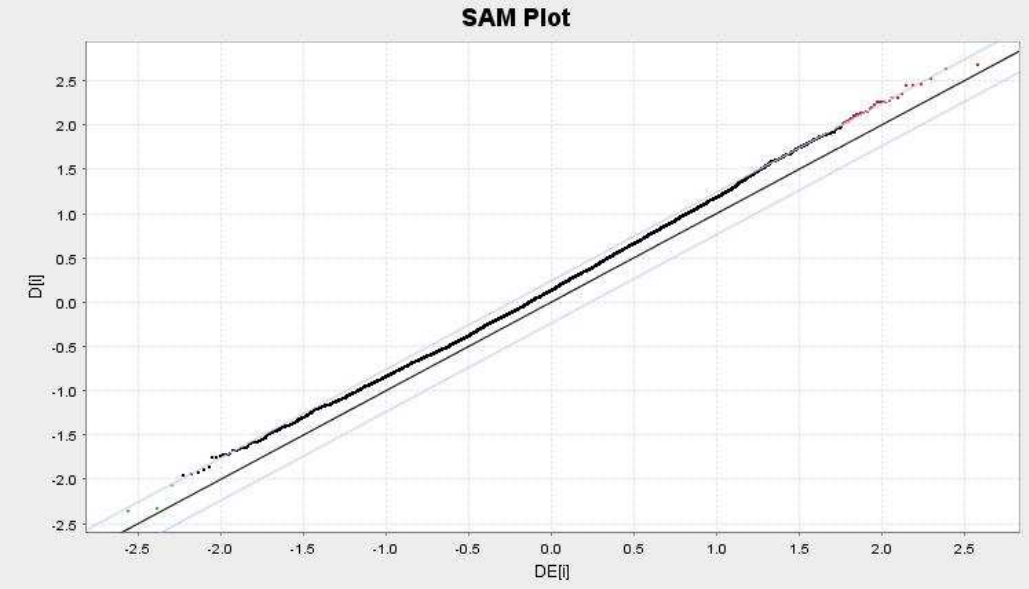


Figure 71 –SAM plot of DD (IF) vs. DD : moderate / poor function /non function (early and late onset), (FDR 0%).

The genes identified in the SAM plot Figure 71 are shown in red and are therefore upregulated in the immediately functioning group compared to the poor or non-functioning group. These genes are listed in table 25 and may therefore be thought to have a protective role.

Accession	Description
NM_014138	Homo sapiens transmembrane protein 29
NM_016292	Homo sapiens TNF receptor-associated protein 1 (TRAP1)
NM_000954	Homo sapiens prostaglandin D2 synthase 21kDa (brain) (PTGDS),
NM_000462	Homo sapiens ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome) (UBE3A)
NM_002372	Homo sapiens mannosidase, alpha, class 2A, member 1 (MAN2A1)
NM_002490	Homo sapiens NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa (NDUFA6), nuclear gene encoding mitochondrial protein.
AK023297	Homo sapiens cDNA FLJ13235 fis, clone OVARC1000304, highly similar to PROTEIN MOV-10
NM_018011	Homo sapiens hypothetical protein FLJ10154 (FLJ10154)

Table 25 - DD (IF) vs. DD : immediate / poor function /non function (early and late) (FDR 0%). Genes shown are upregulated in the immediately functioning group and potentially have a protective role.

3.4.3.2 Hierarchical clustering

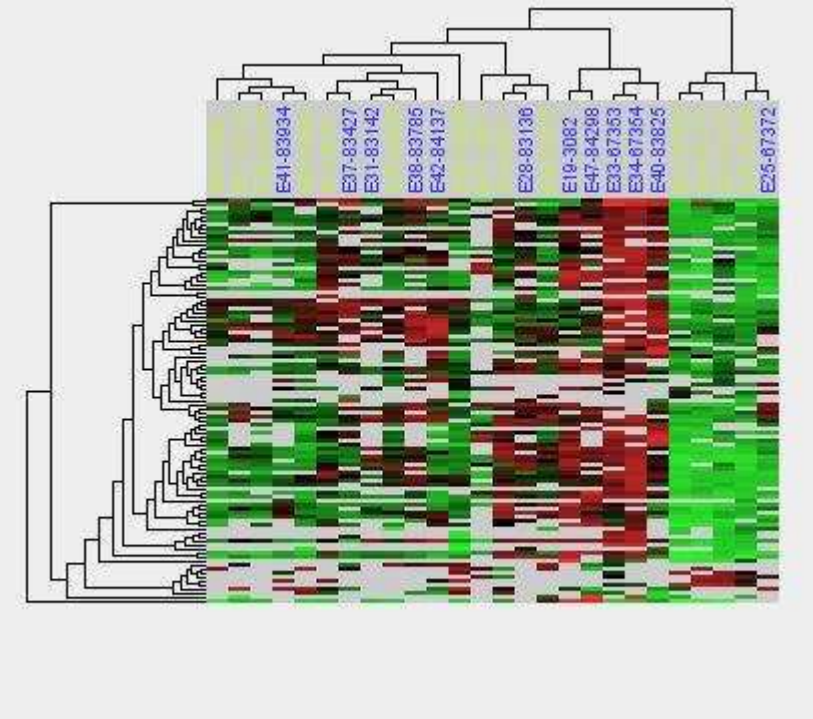


Figure 72 - Hierarchical Clustering: Deceased Donors (Immediate function) versus Deceased Donors
(Moderate / Poor / Non-function)

Hierarchical clustering (Figure 72) shows a degree of sample clustering which is to be expected. Genes are clustered into much smaller clusters. This would fit with the SAM analysis as few genes were shown to be differentially expressed and so unlikely to be clustered together in relation to the rest of the dataset.

3.4.3.3 Gene Ontology / Pathway Analysis

As only a few genes were identified with SAM analysis no further analysis was performed using gene ontology or pathway analysis.

4 DISCUSSION

During this study period a set of 43 kidney biopsy samples were analysed for gene expression using a microarray using the complete human genome from RNA to Cye dye labelled probes. All 43 samples were hybridized successfully with a healthy control kidney sample. For the purpose of data analysis the samples were grouped depending on clinical scenario and donor type. In the main analysis comparing immediately functioning kidneys of deceased vs. living donors 1802 genes were found to be significantly differentially expressed. With a false discovery rate of 0, 190 genes were found to be differentially expressed of which 143 were negatively expressed and 47 were positively expressed.

A proportion of the differentially expressed genes already have a biological function assigned to them and some of these genes have potential as biomarkers. Such biomarkers have the potential to act as surrogate markers of graft function or could be used to monitor the immune reactivity of the graft or recipient. The adverse effects of some of the genes may be modified by the use of pharmaceuticals Other biomarkers have been shown to be modifiable by the use of pharmaceuticals. Other genes were identified that were significantly differentially expressed, however, their gene sequence and biological function have not yet been confirmed. Biomarkers identified by this study, therefore, have the potential to improve the monitoring and treatment of kidney transplant recipients which may improve the long term graft and patient survival.

Between the period December 2000 to May 2005 196 donor samples were assessed for their suitability for microarray analysis. Using good samples, quality controls checks were repeated and also performed by MWG. Despite this, the initial methodology, probes made using small but apparently sufficient amounts of good quality RNA, failed to hybridise.

As a result of these initial studies a new methodology was adopted. The recent application of genome wide microarray analysis along with a newer ‘tried and

tested' methodology was therefore, employed. Compared to the initial methods there were differences in the new method which offered several advantages.

The development of a reliable methodology began with the tissue handling and storage technique. The Agilent Bioanalyser confirmed that tissue stored in RNA*later*® was more stable than when RNA*later*® was not used. The minigel trace of samples demonstrated that the 18 and 28S RNA bands were clearly demarcated indicating RNA was preserved in the majority of samples stored using RNA*later*®. Of the one hundred and ninety six kidney biopsy samples that were taken between December 2000 and September 2005 and were quantitatively and qualitatively, forty seven samples were found to be suitable for microarray and 43 were used for analysis. Of the 47 samples 35 had been initially stored in RNA*later*®. Only 10 of the remaining 149 samples not stored initially in RNA*later*® were suitable for microarray. These findings confirmed the need to use RNA*later*® for storing tissue samples in order to preserve RNA and prevent significant degradation. These findings concur with other authors findings that use of RNA*later*® improves RNA quality and quantity in stored tissue [151].

In the initial method a simple scheme was proposed for the Cye Ester fluorescent dye labelling of samples. For deceased samples the control sample was to be labelled with Cy5 dye and the control samples of living donor samples were labelled with Cy3 fluorescent dye. Since the original experimental design aimed to compare two samples taken from the same recipient at different times the pre-implantation biopsy was labelled with Cy3 and the post-transplantation biopsy with Cy5. Due to the nature of transplantation the supply of samples fitting this design was extremely limited. Further to this, the quality of these samples prior to the use of RNA*later*® was poor, and so this scheme was not applied.

The initial methodology proposed to compare samples taken from the same kidney at different times. E1 was an experiment of this type. It became apparent that the supply of follow-up biopsies taken from the same kidney was

going to be extremely limited. For this reason the data from E1 was not used in the analysis. However, the samples used in E1 were not wasted. The RNA from these samples had already been coupled to their respective dye depending on donor type and so when the use of a control sample was employed in the new strategy the complimentary dye was used. Data analysis software had the ability to take into account which dyes the test or control sample has been coupled to in the initial method direct labelling experiments. In J-express "dye swapping" is a feature that was used to compensate for this [152-154]. The purpose of dye swap recognition was to take into account the slightly increased affinity for Cy3 over Cy5 dye, potentially in the case of a deceased donor experiment, giving falsely elevated spot intensities for the deceased channel. Living donor experiments could therefore appear to have reduced spot intensities i.e. reduced induction / suppression of genes. Dye swapping corrected for these slight differences in cye dye affinity. The new method used indirect labelling for cye dye incorporation in which there is equal labelling and no bias, therefore, dye swapping was not performed.

There were technical differences between the initial method and the new method that had a number of beneficial implications. The amount of total RNA required in new method was 1µg compared to 5µg in the initial method. This allowed for the potential analysis of a larger number of samples where the RNA yield following extraction was very low (i.e. < 5µg). It also allowed hybridisations to be repeated if they had failed. In the initial method several samples were never analysed because all of the extracted RNA was used in the hybridisation, which failed.

There were differences between the initial and new strategies in reverse transcription and dye coupling. In the initial method reverse transcription began at the 3' end of RNA and was performed separately to dye labelling. In the new method antisense RNA (aRNA) was produced compared to sense RNA and dye coupling occurred between the amino allyl modified UTP residues on the RNA and the amine reactive dyes; the initial method produced unmodified aRNA. There was likely to be a difference in the efficiency of

transcription. For the few templates that were transcribed more or less efficiently than other templates in the initial compared to new method, the amplification bias had been shown by the manufacturer to be typically equivalent [155-157]. Even though the aRNA amplification procedure may not have generated exactly the same number of aRNA molecules from each template (sample), amplification had been shown by the manufacturer to be reproducible from reaction to reaction making it possible to compare the expression profiles of different amplified RNA samples [155-157].

The manufacturer had compared RNA polymerase activity in the two methods. They found that it was not affected by either the concentration of individual templates in a complex mixture or by the sequences of the template molecules being transcribed [157], and was shown not to significantly distort the relative abundance of individual mRNA sequences within an RNA population [155-157].

The incubation at the amplification / transcription stage was done in air incubator in the new method (c.f. water bath) which made the possibility of condensation formation in the reaction tubes much less likely. The initial method was, therefore, more likely to produce condensation (an observation that was made) potentially affecting the concentration of the reaction mix during amplification. This could then cause variation in the yield across the batch of samples being incubated at the same time, adding error to the experiment. This hypothesis was not formally investigated because no probes made using the initial technique were successfully hybridized. This fact does however suggest that condensation formation in the reaction may have significant detrimental effects on probe integrity. All incubation stages in the new method were carried out in an air incubator.

Data filtering and Normalisation

It was important to ensure that the data was handled appropriately in order to achieve quality data that was interesting and relevant. There is no defined protocol to follow when handling microarray data. It is up to the researcher to

decide on the best methods / parameters for filtering and normalisation. From the same raw dataset it is possible, therefore, to obtain different resulting datasets depending on which filtering parameters are used. Filtering parameters were set in order to exclude poor quality data, artefact and background 'noise'. Filters were set to extract data representing significant changes in gene expression i.e. above two fold change, between groups. Gene filtering, therefore, excluded genes that did not change. It is possible that if genes involved in a process that is genuinely positively or negatively expressed, are filtered out e.g. because the differential expression was less than two-fold; then the overall process / pathway could be overlooked because only a few genes are identified within it.

Arbitrary cut-off values were made when setting the filtering parameters. These were based on recommendations made by other laboratory workers' experience of the technique and based on the J-express user guide [158]. The value filters were used to exclude data with extreme expression values deemed to be artefactual. The values that were set were based upon the expression values and therefore potentially could have excluded good data with high expression values. The other filters based upon recommendations [158] also had the theoretical potential to exclude some good expression data, an unavoidable reality of microarray analysis.

Another potential limiting factor was overall number of samples that were used in data analysis. Relatively small numbers of samples fitting a specific clinical scenario e.g. follow-up biopsy meant that that particular clinical scenario could not be analysed as separate experiment, thus smaller groups of donor samples were grouped together for analysis.

Due to the nature of the experiment tissue samples were not abundant. From an overall pool of 196 potential donor samples only 47 were suitable for analysis and 43 used for analysis. The small quantities of starting RNA limited the number of experiments and did not allow replicate experiments to be

performed. To some extent the latter point is compensated by the intra-experimental replication of probes i.e. duplicates on the microarray slide.

Another drawback of having limited tissue samples was this did not allow for any further analysis techniques to validate expression levels such as quantitative real-time PCR. The aim of the study, however, was not to measure exact gene expression levels, but to look for novel genes, groups of genes or pathways that could be further investigated and validated by RT-PCR. In contrast to other quantitative studies this study had many potential clinical scenarios to investigate and compare to the common control which would hopefully produce biologically and clinically relevant data.

In spite of the limitations this study generated a large number of data points which allowed further analysis in relation to clinical parameters.

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4.1 Clinical scenarios

Data was analysed following normalisation using Significance analysis of microarrays (SAM) and Hierarchical clustering. The resulting datasets were then used in Gene Ontology Mapping and Pathway Analysis to investigate the distribution of significant genes and hence which biological processes were being activated or repressed. The main analyses were performed depending on clinical scenario:

A. Deceased Donors (Immediate function) versus Living Donors (Immediate function)
B. Deceased Donors (Immediate function) versus all Donors (Moderate / Poor / Non-function)
C. Deceased Donors (Immediate function) versus Deceased Donors (Moderate / Poor / Non-function)

Significance analysis of microarrays (SAM) is a statistical testing algorithm which identifies induced and repressed genes with significantly different expression across samples [144]. It compares 2 or more samples normalised to the control sample. SAM was used to identify significant genes based on differential expression between groups of samples created depending on the clinical scenario.

Hierarchical clustering is a technique in which each gene is assigning to its own cluster [145]. The closest (most similar) pair of clusters is merged into a single cluster, so that there is one less cluster. Distances (similarities) are computed between the new cluster and each of the old clusters. This process is repeated until all items are clustered into a single cluster of size N.

Gene ontology is a collaborative research tool developed to construct and use ontologies to facilitate the biologically meaningful annotation of genes and their products [159]. GO annotations have already assigned biological function to genes which are subsequently grouped by process e.g. Apoptosis and are

organised and related in a hierarchical manner. Therefore, genes involved in the same process with similar / different expression patterns are examined.

Pathway analysis applies microarray data e.g. from SAM analysis and overlays this data onto a database of molecular networks assembled from scientific papers. It is a tool to visualise data in a biological sense and to see which processes significant genes are involved in and how they interact with each other and other processes. Another application of pathway analysis involves the use of Medscan. The Medscan tool was useful for exploring and visualising data. It uses a dataset (e.g. from SAM) and looks for publications relating it to user defined search terms. The resulting pathway is built and each link can be clicked on, to link the investigator to those publications.

4.1.1 Deceased Donors (Immediate function) versus Living Donors (Immediate function).

We know that deceased donor kidneys have higher rates of graft dysfunction and failure in the long term [160]. The purpose of this analysis, therefore, was to identify genes and processes that have the potential to explain this phenomenon. The main issues were to examine the differences between grafts from the two donor sources to see if there are any processes that differentiated between the two groups.

The mechanisms underlying the differences in outcome include the following:

1) The damage caused to the deceased donor kidney by brainstem death is likely to reduce the number of functional nephrons This may manifest itself as a graft that has reduced function from the outset or it may have normal function initially which deteriorates at a faster than expected rate.
2) Brain-stem death, hypoxia and warm and cold ischaemia may cause activation of pathological processes that cause damage to the graft. This may occur immediately and result in a poorly functioning graft or may

occur over time, accumulating, resulting in chronic allograft nephropathy; a known cause of graft dysfunction, deterioration and loss.

SAM detected 190 differentially expressed genes of which 143 were down-regulated and 47 were up-regulated. More genes were down-regulated suggesting that the consequences of brain stem death / ischaemic damage are as a result of the down-regulation of protective cellular functions.

4.1.1.1 Upregulated Genes.

A set of genes was upregulated in the deceased donor group compared to the living donor group. The amount of up-regulation was represented as the fold change. In this set of genes the range of fold change was between 2.2 and 81.7 times. At a cellular level the majority of genes functioned at the cell and plasma membrane, within the cytoplasm, mitochondria and within the nucleus. Molecular functions involving binding, transporter and catalytic activity and biological processes involving cellular physiological and communication processes contained the majority of up-regulated genes.

Deceased donor kidneys have been shown to have a poorer 10 year graft survival rate [160]. Although the rate of graft survival has improved for both groups there still remains a clinically significant difference in graft survival and this depends on whether or not the kidney graft was sourced from a living or deceased donor.

Long-term graft failure can be secondary to chronic allograft nephropathy (CAN), recurrent renal disease and death from another cause with a functioning graft. CAN is secondary to a combination of chronic rejection, chronic cyclosporine toxicity, and/or donor kidney disease[161]. The genes that were identified therefore potentially indicate processes that were up-regulated in the deceased donor kidneys and not the living donor kidneys, i.e. damaging processes. These genes may also represent genes that were up-regulated, but to a lesser degree (of fold change) in the living donors, or represent genes that

were down-regulated in the living donors and, as a result of data normalisation, the actual value was expressed as a positive value.

The first explanation (genes up-regulated in deceased donors) is more likely; however, the actual values or ratios given for each gene are not necessarily important, it is the identification of the process that may be of potential clinical relevance.

The following genes of known function which may be relevant to transplantation were up-regulated in deceased donors.

i. Homo sapiens major histocompatibility complex, class II, DR beta 4 (HLA-DRB4)

HLA-DRB4 was positively expressed 22.3 times more in the deceased donor samples. HLA-DRB4 mRNA belongs to the HLA class II beta chain paralogues [162]. It is a class II heterodimeric molecule which consists of an alpha and a beta chain anchored to the cell membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (B-lymphocytes, dendritic cells and macrophages). Within the DR molecule the beta chain contains all the polymorphisms specifying the peptide binding specificities. Typing for these polymorphisms is routinely done for bone marrow and kidney transplantation.

HLA-DRB4 has been shown to have an association with Type 1 diabetes mellitus, the insulin gene, and CTLA4[163]. It has an associated with the development of vitiligo an autoimmune condition affecting the skin [164]. It has also been shown to have an association with sinonasal polyposis [165].

The clinical significance of these findings is that there is an auto-immune element that is more likely to be induced in the deceased donor group. Chronic allograft nephropathy is a condition that causes long term deterioration in graft function and graft loss and represents cumulative and incremental damage to

nephrons from time-dependent immunologic and non-immunologic causes [166].

Interestingly tacrolimus immunosuppression is more commonly given to living donor recipients and has been shown to be more diabetogenic than cyclosporin [167]. The finding that HLA-DRB4 is upregulated in the deceased donors would suggest that there should be a higher incidence of diabetes among this group. This hypothesis is however, complicated by the fact that many deceased donors are converted to tacrolimus when they exhibit evidence of progressive deterioration in renal function [125]. Further analysis of the diabetic status of patients was outside of the scope of this study.

Patients who developed new onset diabetes after transplantation have been shown to have a remission rate of 42% following conversion from tacrolimus to Cyclosporin. Renal graft and patient survival rates were equivalent to the group that remained on tacrolimus. There was no increase in the rate of chronic rejection[168] .

The clinical implication of these findings is that if HLA-DRB4 expression is seen to be high then cyclosporine immunosuppression would be the preferred choice of immunosuppression, over the long term, for both deceased and living donor recipients.

ii. Homo sapiens tumour necrosis factor (ligand) superfamily, member 10 (TNFSF10), mRNA.

This gene was up-regulated by 81.7 fold. The protein encoded by this gene is a cytokine that belongs to the tumour necrosis factor (TNF) ligand family and is also known as Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL). This protein binds to several members of TNF receptor superfamily. It preferentially induces apoptosis in transformed and tumour cells, but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues[169]. Song *et al* performed immunohistochemical staining of rejected compared with normal renal tissue. TRAIL was strongly expressed

both in the cell membrane and plasma of renal proximal and distal convoluted tubules of rejected tissues [170].

TRAIL was significantly upregulated in the deceased donor group compared to the living donor group. This would suggest that cells expressing TRAIL are susceptible to apoptosis and would explain why its increased expression in the deceased donor group is associated with a poorer outcome secondary to increased rejection episodes, graft deterioration and loss. It therefore has a potential as a biomarker for apoptosis and graft damage. Patients in whom TRAIL was raised would require closer monitoring, possible biopsy to exclude a reversible cause and consideration of change in immunosuppression.

iii. Proteolipid protein 2 (PLP2)

PLP2 was up-regulated 14.7 fold. It is a chemokine that binds to CCR1, which plays important roles in immune and inflammatory responses. Over expression of PLP2 stimulated a twofold increase in the agonist-induced migration of Human osteogenic sarcoma (HOS)/CCR1 cells, implicating a functional role for PLP2 in the chemotactic processes via CCR1 [171].

PLP2 may therefore have a role in the monitoring of the immune / inflammatory response that follows transplantation or used to assess the response at any time of graft dysfunction. It would therefore alert the physician to closely monitor graft function and intervene at an earlier stage.

4.1.1.2 Down-regulated genes

A set of genes were down-regulated in the deceased donor group compared to the living donor group. The amount of down-regulation was between -2.7 and -235 times. The overall functions of these genes are the same as those functions of the up-regulated genes. Gene ontology does not differentiate between up and down-regulated; it merely identifies the differentially expressed process. Therefore, at a cellular level the majority of genes functioned at the cell and plasma membrane, within the cytoplasm, mitochondria and within the nucleus.

Molecular functions involving binding, transporter and catalytic activity and biological processes involving cellular physiological and communication processes contained the majority of down-regulated genes.

The genes that were identified were down-regulated in the deceased donor group; however, they could also potentially indicate processes that were not changed in the deceased donor group but were expressed at higher levels in the living donor group giving an overall negative value of different expression, If this is the case then the processes identified could be seen as 'protective'. These genes may also represent genes that were up-regulated in the deceased donors group, but to a lesser degree (equal to the fold change) compared to the living donor group, hence have a negative differential value. As before the first explanation is more likely, however, it is the identification of the process that may be of potential clinical relevance.

The most negatively differentially expressed genes found in the dataset were:

i. Homo sapiens glutamine and serine rich 1 (QSER1), mRNA

This is a theoretical gene. It was negatively expressed in the deceased donor group by 235 fold. No reports of its function are found in the literature. It is of interest, however, and is an area of potential further research. Its use would be as a surrogate marker of graft function.

ii. Homo nephrosis 1 homolog, nephrin (human) (NPHS1)

NPHS1 was negatively expressed by 160 times. This novel gene has been found to be defective in patients with congenital nephrotic syndrome of the Finnish type (CNF) leading to abnormal expression of the respective protein product nephrin in glomerular cells [172]. CNF patients treated with nephrectomy and renal transplantation have a 20% chance of recurrence of nephrotic syndrome (NS). Wang *et al* found an increased antibody titre to nephrin prior to the NS [172]. There results showed that a transplantation induced production of autoantibodies occurred, pathogenic to the glomerular filtration barrier function. This gene was significantly down-regulated in the deceased group suggesting that down-regulation of the normal gene may lead

in the long term to abnormal function of the glomerular membrane. Nephrin therefore, could be used as a surrogate biomarker where reduced titres would alert the physician to the potential development of nephrotic syndrome and graft deterioration.

The recurrence of proteinuria following transplantation is associated with allograft glomerular pathology[173], and gives a theoretical explanation of why reduced expression of the normal gene leads to a pathological deterioration in graft function.

iii. Homo sapiens cell division cycle 6 homolog (CDC6), mRNA.

CDC6 was down-regulated 92 fold. The protein encoded by this gene is essential for the initiation of DNA replication[174]. It functions as a regulator at the early steps of DNA replication and localizes in the cell nucleus during cell cycle G1, but translocates to the cytoplasm at the start of S phase. Lau *et al* showed that depletion of Cdc6 in synchronous G1 cells blocks G1 to S transition, confirming the role of Cdc6 in the initiation of DNA replication. In contrast, depletion of Cdc6 in synchronous S-phase cells slowed DNA replication and led to mitotic lethality. These results show that Cdc6 is not only required for G1 origin licensing (the initiation of DNA replication), but is also crucial for proper S-phase DNA replication that is essential for DNA segregation during mitosis.

Significant down-regulation in this gene in the deceased donor may reduce the graft's capacity to regenerate and repair leading to accelerated cell and hence graft loss. It could therefore be used to alert the physician to a graft that is potentially failing in order to institute supportive diagnostic and therapeutic measures, including the modification of immunosuppression.

Hierarchical clustering of significantly differentially expressed genes identified by SAM (FDR 0%) revealed two main groups of genes. Group A refers to genes that were down-regulated in the deceased versus living donor group. Group B refers to genes that were up-regulated in the deceased donor group. In

order to look at these in a more biologically relevant way, pathway analysis was performed with this dataset and correlated with a literature search for transplantation and inflammation using the Medscan search tool within the pathway studio program.

Gene Ontology found that significant data distributed to Cellular components was mainly involved in processes incorporating the cell or plasma membrane and within the cytoplasm and nucleus. Catalytic activity, signal transducer and transcription regulator activity contained the majority of genes within the molecular processes category. Signal transduction, cellular metabolism and response to stimulus (including immune response) contained the majority of genes within the category biological processes.

Gene ontology mapping is not specific enough to indicate which genes are implicated in graft events; this specific data can be found using SAM. Gene Ontology does, however, give a breakdown and visual representation of microarray data and highlight the areas of interest from which we can go back to the raw data to analyse in more detail and can suggest areas where further research should be undertaken.

Pathway analysis identified the following pathways that were significantly differentially expressed between deceased and living donors.

A. Death Receptor Pathway, DR3 / DR4 Pathway , Inhibition of Apoptosis
B. Epidermal Growth Factor Signalling
C. IFN-α / β / γ EGF

Pathway A - Death Receptor Pathway, DR3 / DR4 Pathway and (Inhibition of) Apoptosis Pathway.

Death receptors are cell surface receptors that transmit apoptosis signals initiated by death ligands [175]. They play a central role in apoptosis. Apoptosis is specifically induced via signalling through the tumour necrosis factor (TNF) receptor gene superfamily which includes Fas, TNFR, DR3, 4 and 5. Death receptor ligands characteristically initiate signalling via receptor oligomerization, recruitment of specialized adaptor proteins and activation of caspase cascades [176].

Established death receptors are CD95 (also called Fas or Apo1) and TNFR1 [177] Additional death receptors include death receptor 3 (DR3) and DR4. DR3 promotes apoptosis via the adaptor proteins TRADD/FADD and the activation of caspase 8. The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily [178]. Apo2 ligand (Apo2L, also known as TRAIL) binds to DR4 [179].

Activated caspase 8 stimulates apoptosis via two parallel cascades: it cleaves and activates caspase-3, and cleaves Bid (a Bcl-2 family protein) [180]. DR-3L can deliver pro- or anti-apoptotic signals. DR-3 promotes apoptosis via the adaptor proteins TRADD/FADD and the activation of caspase 8. Alternatively apoptosis is inhibited via an adaptor protein complex including RIP which activates NF-kB and induces survival genes including IAP.

TRAIL was a identified in this study and was fond to be up-regulated by 81.7 fold. The clinical implications are that if pro apoptotic signals such as TRAIL are identified then the physician is alerted and is able to employ supportive

measures should the transplanted graft develop dysfunction. If anti-apoptotic signals are identified then this can be seen as a good prognostic indicator of graft function and survival.

Pathway B – Pro-Epidermal Growth Factor Receptor signalling pathway.
This pathway contained significantly differentially expressed genes. Pro-epidermal growth factor precursor (EGF) was one gene that was identified in the SAM dataset (FDR 5%). It was downregulated by 2.5 fold. The epidermal growth factor receptor (EGFR) signalling pathway regulates growth, survival, proliferation and differentiation in the mammalian cell [181]. The epidermal growth factor (EGF) peptide induces cellular proliferation through the EGF receptor. The proliferative effects of EGF are signalled through several pathways. The EGF receptor activates ras and the MAP kinase pathway, ultimately causing phosphorylation of transcription factors such as c-Fos to create AP-1 and ELK-1 that contribute to proliferation. Activation of STAT-1 and STAT-3 transcription factors by JAK kinases in response to EGF contributes to proliferative signalling.

Smooth muscle proliferation along with lymphocyte proliferation is implicated in chronic allograft nephropathy as a cause of graft deterioration [182]. Mycophenolate mofetil has been shown to reduce the rate of decline in renal function in patients with established chronic allograft nephropathy [183].

The clinical implications of this would be that the detection of differentially expressed genes such as Pro-epidermal growth factor precursor (EGF)
pathway would alert the physician to the possibility of the development or progression of chronic allograft nephropathy. This may prompt a change in immunosuppression to an agent such as mycophenolate mofetil or to the reduction / cessation of calcineurin inhibitor.

Pathway C - IFN-α / β / γ and EGF Pathway.

Interferons have several effects in common. They are antiviral and possess antioncogenic properties. They activate macrophage and natural killer T-lymphocytes and through the major histocompatibility complex present foreign peptides to T cells.

Interferon alpha plays a role in viral infections. Signalling takes place through an IFN Receptor complex consisting of two alpha chains (Type I receptor) complexed with Jak1 and Tyk2. These kinases phosphorylate STAT1 and STAT 2 respectively.

STAT family members are phosphorylated in response to cytokines and growth factors, by the receptor associated kinases, and then form heterodimers that translocate to the cell nucleus where they act as transcription activators. STAT1 can be activated by various ligands including interferon-alpha, interferon-gamma, EGF, PDGF and IL6. The protein mediates the expression of a variety of genes thought to be important for cell viability in response to different cell stimuli and pathogens. Two transcript variants encoding distinct isoforms have been described.[134, 184].

Interferon gamma is secreted from CD4+ Th1 cells, CD8 cells, gamma/delta T cells and activated NK cells. It plays a role in activating lymphocytes to enhance anti-microbial and anti-tumour effects. In addition it plays a role in regulating the proliferation, differentiation, and response of lymphocyte subsets. Signalling takes place through the IFN receptor complex.

The clinical significance of monitoring expression of the interferons is likely to be limited. They play a complex role in the cytokine network and may therefore have a more important role in the monitoring of the immune or inflammatory responses but whose actions appear to be too generalised to be of any specific monitoring or predictive value.

4.1.2 Deceased Donors (Immediate function) versus all donors (Deceased and Living), with Moderate / Poor / Non-function.

The purpose of this sub-analysis was to potentially identify genes or groups of genes that were differentially expressed in deceased donor kidneys that functioned well from the outset to all other kidneys from both sources that did not. This analysis would potentially indicate processes that were present in the graft at the time of biopsy prior to the process becoming apparent clinically. Differential expressed processes may also be caused by the difference in donor source.

Kidneys that did not function immediately or at all did so for a number of clinical reasons:

- Delayed graft function
- Vascular thrombosis
- Acute Rejection
- Drug Toxicity
- Unknown

This analysis would not be able to differentiate between the above clinical scenarios as causes of graft dysfunction; to do so would create much smaller groups, the sub-analysis of which highly unlikely to be of statistical or clinical significance.

Supporting data was identified by SAM (FDR 5%) in the deceased donor immediate function vs. non-immediately functioning kidneys. SAM detected 109 significantly differentially expressed genes. Hierarchical clustering of these genes did not show any grouping of genes related to donor type (deceased or living).

Gene ontology revealed identified genes to be involved in cell membrane, nucleus and cytoplasm activities. The rest of the significantly differentially expressed genes were spread over many processes. Many of the cellular processes involved were happening in the cytoplasm. Binding and catalytic processes were the predominant processes occurring under the term molecular function. Cellular metabolism was represented with the most number of

differentially expressed genes. These finding are not unexpected and represent the fact that different processes were occurring in the cytoplasm of kidneys that functioned well compared to those that did not.

Tumour necrosis factor type 1 receptor associated protein (TRAP 1) was down-regulated by 2.2 fold. It has been found to be suppressed by beta-Hydroxyisovalerylshikonin (beta-HIVS), a compound isolated from the traditional oriental medicinal herb Lithospermum radix and VP-16 an inhibitor of the enzyme topoisomerase II [185]. They are ATP non-competitive inhibitors of protein-tyrosine kinases, such as EGFR, and induce apoptosis in various lines of human tumour cells. The suppression of the level of TRAP1 by beta-HIVS or VP16 was blocked by N-acetyl-cysteine, indicating the involvement of reactive oxygen species (ROS) in the regulation of TRAP1 expression. These results suggest that suppression of TRAP1 expression in mitochondria may have an important role in the induction of apoptosis caused by the formation of reactive oxygen species [185].

Im *et al* found that deferoxamine (DFO) treatment to TRAP1-overexpressing cells resulted in decreases in levels of ROS, Cav-1, glutathione peroxidase (GPX), manganese superoxide dismutase (MnSOD) levels and senescence-associated beta-galactosidase (SA beta-gal) activity. These results suggest that TRAP1 may play a role in protecting mitochondria against damaging stimuli via decrease of ROS generation [186] .

These finding are in keeping with the clinical scenario; possibly identifying processes which explain a difference in kidneys that function well from the outset (immediate function) to those that do not. Therefore TRAP-1 has the potential to be a biomarker for apoptosis and/or mitochondrial damage. Also, TRAP-1 expression has been shown to be suppressible by beta-HIVS, VP16 and DFO thus indicating a possible therapeutic role.

TRAP-1 was not recognised by Gene ontology mapping. The reason for this is likely to be that although the record has had preliminary review of the

sequence by NCBI Reference Sequences (RefSeq) it has not yet been subject to final review.

The other main groups of genes belonged to cellular and biological processes that are of unknown function. These genes are of interest as they may represent novel genes that could have the potential to serve as biomarkers.

Pathway analysis identified a number of genes. One of those was the RET (rearranged during transfection) proto-oncogene. This is a member of the cadherin superfamily, which encodes one of the receptor tyrosine kinases cell-surface molecules that transduce signals for cell growth and differentiation. It is associated with the development of various tumours.

Another oncogene, BCL3 identified by pathway analysis has recently been described by Kusaka *et al*. They performed global expression analysis on brain dead rats (equivalent to deceased donors) and found BCL3 to be one of 22 upregulated genes. They postulated that the presence of these genes may pose as novel target genes for treatment and prognosis of grafts from brain-dead donors [187].

CD-63 has been used as a marker of response to treatment with the platelet activation inhibitor Lipo PGE1 during acute rejection episodes. Zhang *et al* randomly assigned forty patients with acute rejection after kidney transplantation into groups treated with or without Lipo PGE1. Compared with controls, the expression levels of CD61, CD63, and PAC-1 were lower among acute rejection patients who received Lipo PGE1 therapy. Their 1-year patient and graft survival rates were higher. They concluded that Lipo PGE1 therapy in patients with acute rejection episodes may benefit graft functional recovery via the inhibition of platelet activation [188].

Results found in this thesis corroborate other authors' findings. Interestingly CD-63 has been used as a biomarker that is measured in the peripheral blood.

This adds evidence to suggest that novel genes identified by microarray analysis may have a diagnostic and monitoring role in transplantation.

Prostaglandin D synthase also known as ß-Trace protein (BTP) was downregulated by 1.4 fold. It is a 23- to 29-kDa enzyme that has been proposed as an alternative marker for GFR in children and in people with diabetes or various renal diseases [189], The protein encoded by this gene is a glutathione-independent prostaglandin D synthase that catalyzes the conversion of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2).

Based on the authors results, they believe that serum levels of Prostaglandin D synthase may be a useful and reliable biomarker to estimate GFR[190]. The implication here is that microarray analysis can identify biomarkers that can also be measured in serum to estimate and monitor graft function.

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SPP1 (also known as Osteopontin and early T-lymphocyte activation 1 (Eta-1)) has been shown to influence the type I immune response. Ashkar *et al* identified Eta-1 as a key cytokine integral to efficient type-1 immune responses through differential regulation of macrophage IL-12 and IL-10 cytokine expression [191]. Cell-mediated immunity is necessary for immune protection against most intracellular pathogens. When excessive it can mediate an organ-specific autoimmune response. Autoimmunity has been implicated in the development of chronic allograft nephropathy [192]. Sirolimus is an immunosuppressant with potent antiproliferative actions and represents a therapeutic option in the prevention of acute renal allograft rejection and chronic allograft nephropathy [193].

The implications of these findings are, therefore, that SPP1 has the potential as a biomarker to indicate the level of activity of the immune response and where appropriate, the use of immunosuppression can modify this response in the hope that acute rejection and chronic allograft nephropathy can be prevented or reversed, so improving the function and longevity of the graft.

4.1.3 Deceased Donors (Immediate function) vs. Deceased donors with Moderate / Poor function / Non function

Being sourced from the same type of donor these kidneys should have, in theory experienced similar events preceding transplantation. The difference between this analysis and the analysis in scenario B is that all those factors that could be explained by brain stem death and the autonomic insult are the same for both groups. This should, therefore, highlight different processes unique to one or other group that may explain why otherwise similar kidneys have very different outcomes. Upregulated processes in the deceased donor group with immediate function would be deemed 'protective', whereas downregulated processes would be thought to be 'damaging'. Conversely an upregulated process in the deceased group with poor or non-function would be deemed as 'damaging' whereas a downregulated process may have a 'protective' role. The mechanism behind this difference in clinical outcome may involve pre-existing donor and recipient factors, environmental and unknown factors.

SAM analysis of the group DD (IF) vs. DD immediate / poor function /non function (early and late) (FDR 0) produced two genes in common with the analysis of the group DD (Immediate function) versus all donors (Deceased and Living) with Moderate / Poor / Non-function. This finding is to be expected as in the case of this analysis the same dataset is being analysed minus data taken from living donor samples. Thus some of the pre-transplantation factors are removed.

The genes prostaglandin D2 synthase and TNF receptor-associated protein 1 (TRAP1) were significantly differentially expressed in this analysis. As data from the living donor group was removed it is likely, therefore, that these genes were differentially expressed in deceased donors that had immediate function and deceased donors that did not.

This suggests that TRAP1 suppression may lead to a reduction in reactive oxygen species suppression and hence apoptosis in the deceased donor group

causing graft dysfunction and loss. Whether or not changes in expression of TRAP1 and its potential for monitoring and therapeutic options, are limited to deceased donor recipients, is a further area for potential research. With regard to Prostaglandin D synthase its role in monitoring renal function in living donors would also be a subject for further research.

Homo sapiens transmembrane protein 29 is a novel protein that was down – regulated by 10.5 fold. It's function has not yet been described. Homo sapiens NADH dehydrogenase (ubiquinone) was down-regulated 15.2 fold. It has been associated with a systemic lupus erythematosus like disease (an autoimmune disease) in the mouse. No human function has yet been described. The gene sequence for the protein Homo sapiens cDNA FLJ13235 fis, clone OVARC1000304, highly similar to PROTEIN MOV-10 has been described, however no function has as yet been found. Homo sapiens hypothetical protein FLJ10154 is another gene found to be differentially expressed in this dataset that is awaiting confirmation of its gene sequence and has no known function. These genes may therefore have a role as potential biomarkers that should be investigated in future research.

4.1.4 Comparison of genes identified between groups

Comparison of Deceased Donors (Immediate function) versus Living Donors (Immediate function) – Scenario A and Deceased Donors (Immediate function) versus Deceased Donors (Moderate / Poor / Non-function) - Scenario C

It is interesting to observe that far fewer genes were found to be significantly differentially expressed in this analysis of Scenario A than in the analysis of Scenario C. The essential difference between the analyses was that the comparison group in the first analysis were samples that were also functioning well. The comparison group in Scenario C contained samples from donors whose kidneys functioned poorly. It may be expected that many genes and processes would be identified as being differentially expressed in these very different groups. However, it may be the case that the small numbers of gene identified reflects the possibility that the poor and non-functioning kidneys

were damaged to such an extent that normal cellular processes were not occurring and therefore not expressed. Another explanation is that the data representing those genes that would account for the poor function of kidneys was filtered out in the data processing stage.

4.1.5 Urinary Markers / Peripheral Blood Markers.

Although this study did not attempt to identify biomarkers using any methods other that microarray, it did find upregulated pathways containing genes that other authors have found can be used as non-invasive biomarkers. With regard to predicting the presence of acute rejection there is accumulating evidence that non-invasive immune monitoring may be useful in the early period after renal transplant Muthukumar *et al* found that the measurement of FOXP3 mRNA in urine could be used as a non-invasive means of improving the prediction of outcome of acute rejection in renal transplants [194]. Mas *et al* measured transforming growth factor (TGF), epidermal growth factor receptor (EGFR) and angiotensinogen (AGT) in urine and peripheral blood samples from the patients with chronic allograft nephropathy. They found that gene expression levels of the studied markers in urine samples were more representative of the gene expression in the kidney biopsies than in the peripheral blood samples. Magee *et al* used flow cytometry to quantify cytokine production in peripheral blood cells of renal transplant recipients [195]. The technique was sensitive enough to detect differences between transplant recipients and healthy controls but could not find differences between recipients with or without CAN. Recently proteomics has been used in the development of urine biomarkers for detection of renal allograft rejection [196].

This analysis has found many significantly differentially expressed genes across many pathways, some of which have been investigated by other authors and found to have a potential role as urinary biomarkers. Further consideration of the results of this analysis should be made to assess the potential of significant genes already identified as urinary biomarkers. Ideally further studies should involve the use of microarray in conjunction with urine analysis.

5 CONCLUSIONS AND FURTHER RESEARCH

It is usual that a patient experiencing acute graft dysfunction is subject to hospitalisation, multiple blood tests and a transplant biopsy. The biopsy procedure carries significant morbidity [197] and even mortality.

This study has identified potential biomarkers which may facilitate the detection and monitoring of graft dysfunction and guide treatment strategies in terms of modification of immunosuppression and other supportive measures.

A number of potential biomarkers were identified in this study. They may have a role in monitoring the immune / inflammatory response central to the graft /host response in transplantation. They include molecules which have been shown to indicate graft dysfunction in terms of general (e.g. apoptosis)[179] and specific (e.g. nephrotic syndrome) disease processes[172] . They may also have a role in monitoring disease processes that have multiple aetiologies (e.g. chronic allograft nephropathy)[183], or in monitoring the grafts capacity to recover its function [188]. Many significantly differentially expressed genes were identified that are not as yet fully described in terms of structure and function but may have a role as potential biomarkers. There is also evidence to suggest that the process underlying the expression of some potential biomarkers identified can be influenced by pharmaceuticals [185, 186], thus producing a change in its expression, and therefore, such biomarkers may find application in monitoring therapeutic regimes.

It remains to be seen whether or not the use of biomarkers can fulfil these roles and be used clinically to predict and monitor graft function, to inform patient management and have a positive effect on the function and longevity of the graft.

Further Research

This study has found many interesting genes, some of which may have roles as biomarkers. The realisation of these findings will clearly require validation and warrant further investigation.

In addition, further genes of interest may be identified by the use of other analysis methods. The microarray contained approximately 30,000 genes and the dataset analysed contained 1802 genes, through the use of one or a combination of user defined filters. Adjusting the filters would therefore give different or larger datasets which may contain useful data.

Since the beginning of these studies, microarray technology has advanced further. It is now possible to have a four-channel array i.e. one control and 3 test samples. This would allow the analysis of, for example, the pre-transplantation biopsy, a biopsy taken at the time of acute rejection and a protocol biopsy taken after 6 months. As there would be far less opportunity for inter (or in this example, intra) experimental variation, the data would require far less stringent filtering and normalisation and, therefore, maximise the amount of data suitable for analysis.

One disadvantage of studying the gene expression of kidney allografts is that the tissue samples that are obtained by biopsy may contain all or fewer component parts of the kidney (e.g. capsule, cortex, medulla, collecting system etc). It may be that the important processes only occur in specific areas. Laser micro-dissection is a new technique that can dissect specific areas of tissue such as the glomerulus for gene expression analysis [149].

In order to confirm and extend the findings of this study it would be necessary to expand the sample set studied. The experimental design that is chosen should be tried and tested. It should include other analyses such as real-time PCR and Western Blotting to validate / quantify the results. Immunohistochemistry and in-situ hybridisation have a role in identifying where biomarkers are expressed at a cellular and tissue level. Laser micro-

dissection could be used to obtain these specific tissues of interest. It would be reasonable to include the use of a four channel microarray for the reasons outlined above.

Proteomics is a developing technique that has been shown to be a valuable tool for biomarker discovery [196]. There is currently much interest in the development of urine biomarkers for detection of renal allograft rejection as an alternative to needle biopsy. In comparison to microarray analysis (genomics), proteomics is more complicated because the proteome is variable and constantly changing. The apparent discrepancy between the number of genes in the genome and number of proteins in the proteome (~30,000 vs. ~500,000) can be explained by mechanisms such as alternative splicing, protein modification and degradation. Hence products of transcription may give rise to more than one protein or may experience a post-translational modification such as phosphorylation or may interact with other protein molecules. Furthermore, gene expression does not necessarily reflect the level of protein transcription and vice versa.

The targeted use of proteomics in the development of urinary biomarkers has recently been described [196, 198]. Its role in global molecular analyses would appear to be more limited in its current stage of development. The determination of biomarkers in transplantation probably requires a combination of both approaches.

Once biomarkers have been identified and validated, further in vivo studies using animal models would be required to further validate them prior to commencing human studies. Human studies would need to be undertaken ideally in the form of a randomised controlled clinical trial. The design of such a trial would depend on the type of biomarkers identified and the way in which they are to be measured. For example, the control group would have standard care. The test group would have in addition, biomarker monitoring and its predetermined changes to protocol based on biomarker expression, to see if the use of measuring biomarkers in kidney transplantation has a real effect.

Such a study should be co-ordinated by a central body or bodies such as the British Transplantation Society, and should be performed in centres with a proven track record in kidney transplantation and an established microarray facility. There should be an agreed experimental protocol and standards set.

New legislation is currently going through Parliament making consent to organ donation presumed unless a decision to opt out is actively made. This will go some way to address the supply and demand mismatch however, it is hoped that the results of these and future studies contribute to improving graft survival.

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